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14. ABSTRACT

We proved the major hypothesis driving this project that effective regression of breast cancer growth and metastases can be achieved by suppressing tumor angiogenesis with an orally delivered DNA-based vaccine carried by double attenuated Salmonella typhimurium. For this approach, the murine vascular endothelial growth factor receptor-2 (FLK-1) proved to be an effective target. We also targeted the inhibitor of apoptotic protein, surviving, and endoglin and achieved a similar protection against lethal tumor cell challenges. Our data demonstrate that the major mechanism involves MHC-Class I restricted CD8 T cell-mediated killing of proliferating endothelial cells. The use of anti-angiogenic cytokine IL-18 and targeting of tumor-associated macrophages (TAMs) also proved the anti-tumor efficacy of our anti-angiogenic vaccines. We also found that there are no serious the side effects of our DNA vaccines, thus providing the basis for their future clinical application. In fact, the FLK-1 vaccine is in its final preparation for Phase I clinical trials. Our research findings were also adapted by other investigators and extended to therapies of other diseases such as arteriosclerosis. Overall, research funded by this award was successful in providing new strategies for immune therapies of both breast carcinoma and other diseases.

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INTRODUCTION:

Breast cancer kills over 40,000 women annually in the US alone, largely due to metastasis or recurrence of the primary tumor. A successful treatment leading to the eventual elimination of breast cancer represents a considerable research challenge since there is currently no cure for locally advanced or metastatic breast cancer. It is now well established that angiogenesis plays a key role in the growth of solid tumors and their invasion and metastasis. In fact, angiogenesis is a rate-limiting step in tumor development as tumors have been limited to a growth of 1-2 mm^3 in the absence of a blood supply. Beyond this minimum size, the tumor becomes necrotic and apoptotic. Based on these findings, a different approach to tumor immunotherapy that does not target tumor cells directly involves efforts to inhibit tumor angiogenesis, pioneered by Folkman and colleagues and further extended by many investigators. Such approaches rely mainly on the use of specific chemical or biological inhibitors of angiogenesis, requiring their constant administration at relative high dose levels. The objective of this DOD funded project is to induce T-cell mediated suppression of tumor angiogenesis with an orally delivered DNA vaccine carried by attenuated *Salmonella typhimurium* encoding molecules over-expressed by proliferating endothelial cells, such as VEGF receptor 2 (FLK-1), a inhibitor of apoptotic protein-survivin and endoglin (CD105). There are several advantages in targeting the tumor vasculature rather than solely tumor cells. First, endothelial cells are genetically stable and do not downregulate MHC-Class I Ag, an event that frequently occurs in solid human tumors and severely impairs T cell-mediated antitumor responses. Second, immune suppression triggered by tumor cells in the tumor microenvironment can also be avoided by this approach. Third, the therapeutic target is tumor-independent, thus killing of proliferating endothelial cells in the tumor microenvironment can be effective against a variety of malignancies. Finally, proliferating endothelial cells are readily available to lymphocytes in the bloodstream and consequently $CD8^+$ T cells can reach the target tissues unimpaired by anatomical barriers such as the blood-brain barrier or encapsulation of tumor tissues. During the 4 years funded by this DOD grant, we successfully proved the concept that DNA vaccine-induced anti-angiogenic immune responses can suppress breast tumor growth and metastasis. We also defined the immunological mechanisms involved and are in the process of clinical applications of this approach.

BODY:

During the entire research period, successful proof of concept was established for the highly effective anti-angiogenic/anti-tumor effects induced by a DNA vaccine encoding murine VEGF receptor 2 (FLK-1). The results obtained are documented in part in a paper published in NATURE MEDICINE (Appendix 1). Briefly, we demonstrated that an orally applied DNA-based vaccine against FLK-1 did exclusively target genetically stable proliferating endothelial cells in the tumor vasculature. This FLK-1 vaccine effectively protected mice from lethal tumor cell challenges and reduced growth of tumor metastases in both prophylactic and therapeutic settings. This protection was achieved by specific CTL-mediated killing of proliferating endothelial cells in the tumor vasculature, indicating the breakage of peripheral tolerance against the FLK-1 self-antigen. Effective angiogenesis in the tumor vasculature was suppressed without any impairment in fertility, neuromuscular performance or hematopoiesis and only a minor time delay in wound healing. The approach of combining the FLK-1 vaccine with CD40 ligand trimer was abandoned because it would complicate our mechanism studies.

Mini-gene vaccines were constructed to identify specific FLK-1 CTL epitopes. Several minigene vaccines encoding multiple H-2D^d or H-2K^d-restricted nona-peptides proved to be somewhat effective against D2F2 tumor cells challenges, but their relatively low efficacy and lack of specificity obviated further studies. Instead, we identified a single H-2D^b restricted CTL epitope-FLK$_{400}$ (VILTNPISM) in C57BL/6 mice (Appendix 4). By identifying this epitope from 6 H-2D^b or H-2K^b-restricted nona-peptides, we demonstrated that FLK$_{400}$-specific CTL responses were able to kill FLK-1$^+$ endothelial cells, suppress effective angiogenesis and protect mice from EO771 breast carcinoma challenges. Importantly, DNA vaccine encoding the entire FLK-1 gene also induced a FLK$_{400}$-specific response, attesting to the significance of this epitope.

To further prove the concept of anti-angiogenic DNA vaccines' suppression of tumor growth, we also targeted other molecules over expressed by proliferating endothelial cells in the tumor vasculature, such as endoglin and survivin. Endoglin is over-expressed by proliferating endothelial cells and mostly absent on tumor cells themselves. Our data demonstrated that a DNA vaccine targeting endoglin inhibited angiogenesis and protected mice from D2F2 breast tumor cell challenges via CD8 T cell-mediated eradication of endoglin$^+$ endothelial cells (Appendix 10).

Survivin is an inhibitor of apoptosis and is over-expressed by essentially all solid tumors as well as by proliferating endothelial cells. Our data did demonstrate that a DNA vaccine encoding survivin and chemokine CCL21 induced enhanced killing of both tumor cells and proliferating endothelial cells, resulting in suppression of angiogenesis and ultimately protection of mice from lethal tumor cell challenge (Appendix 3). We also tested a DNA vaccine encoding survivin and the NKG2D ligand-H60 (appendix 5 and 6). In this regard, the NKG2D receptor is a stimulatory lectin-like receptor expressed on natural killer (NK) cells, activated CD8$^+$ T cells, γδ T cells and activated macrophages. It mediates co-stimulatory signals for CD8$^+$ T cells and stimulatory signals for NK cells and macrophages. We could demonstrate that the survivin/H60 vaccine effectively activates both the innate and adaptive immune responses, partly due to the increased crosstalk between lymphocytes.

Other anti-angiogenic/anti-tumor approaches were also adopted by DNA vaccines against breast cancers. In this case, IL-18 was used because of its anti-angiogenic effect, together with the transcription factor Fos-related antigen 1 (Fra-1), which is overexpressed on highly aggressive metastatic breast cancer cells. This vaccine by co-expressing IL-18 and Fra-1 was highly effective against breast cancer metastases by inducing activation of T-and NK cells as well as anti-angiogenesis (Appendix 2).

Finally, we also investigated the possibility of inhibiting angiogenesis by targeting cells other than proliferating endothelial cells. Tumor-associated macrophages (TAMs) were shown to play an important role in angiogenesis and are associated with tumor progression and metastasis. We could indeed demonstrate that Legumain, a member of the asparaginyl endopeptidase family functioning as a stress protein, which is overexpressed by TAMs, provides an ideal target molecule for this approach (Appendix 11). In fact, a Legumain-based DNA vaccine served as a tool to prove this point as it induced a robust $CD8^+$ T cell response against TAMs, which dramatically reduced their density in tumor tissues and resulted in a marked decrease in pro-angiogenesis factors released by TAMs such as TGF-β, TNF-α and VEGF. This, in turn, led to a decisive suppression of both, tumor angiogenesis and tumor growth and metastasis.

Key Research Accomplishments:

1. An oral FLK-1-based DNA vaccine exclusively targets genetically stable proliferating endothelial cells in the tumor vasculature. This vaccine suppresses effective angiogenesis and protected mice from lethal tumor cell challenges, and reduced tumor growth and metastases in both prophylactic and therapeutic setting.
2. FLK_{400} (VILTNPISM) is an H-$2D^b$-restricted CTL epitope. FLK_{400}-specific CTL responses killed FLK-1^+ endothelial cells, suppressed effective angiogenesis and protected mice from EO771 breast carcinoma cell challenges. A DNA vaccine encoding the entire FLK-1 gene also induced FLK_{400}-specific responses.
3. Targeting of endoglin, a molecule only over-expressed by the tumor vasculature but not by tumor cells themselves, further proved the concept for the effectiveness of our anti-angiogenic DNA vaccine strategy.
4. DNA vaccine encoding the inhibitor of apoptotic protein survivin together with CCL21 induced MHC-Class I-restricted, CD8 T cells mediated cytotoxicity against both tumor cells and proliferating endothelial cells in the tumor vasculature, leading to tumor protection.
5. A DNA vaccine encoding survivin and NKG2D ligand H60 activated both innate and adaptive immune responses partly due to increased lymphocyte crosstalk.
6. A DNA vaccine encoding Fra-1 and IL-18 effectively protected mice from D2F2 breast tumor cell challenge, by inducing CD8 T cell-mediated cytotoxicity against thesetumor cells, as well as IL-18-mediated suppression of tumor angiogenesis.
7. Targeting legumain-overexpressing TAMs with a legumain-based DNA vaccine induced CTL-mediated specific killing of TAMs, resulting in the suppression of tumorangiogenesis and protection of mice from lethal tumor cell challenges.

Reportable Outcomes:

The reportable outcomes and results from the entire research period covered by this grant are as follows:

1. Six published research articles (Appendix 1-6); 3 published review articles (Appendix 7-9); 1 research article in press (Appendix 10) and 1 research article in revision (Appendix 11).
2. Seven abstracts selected for presentation at AACR annual meetings (Appendix 12-18).
3. Funding applied for based on work supported by this award:
   1 NIH R01 grant: Fra-1: A new target for a genomic breast cancer vaccine.
   1 DOD research grant: An oral DNA vaccine encoding endoglin eradicates breast tumors by blocking their blood supply.
   1 Postdoctoral Fellowship from The Susan G. Komen Breast Cancer Foundation (05, 2003-04, 2006): Blocking Blood Supply to Breast Carcinoma with a DNA Vaccine Encoding VEGF Receptor-2 Flk-1 and NKG2D ligands
4. Currently the FLK-1 vaccine is in preparation for Phase I clinical trial.

Conclusions:

We proved our major hypothesis driving this project that effective regression of breast cancer growth and metastases can be achieved by suppressing tumor angiogenesis with orally delivered DNA vaccines carried by double attenuated *Salmonella typhimurium* to a secondary lymphoid organ, i.e. Peyer's patches. We also demonstrated that the major mechanism involves MHC-Class I restricted CD8 T cell-mediated killing of proliferating endothelial cells in the tumor vasculature. Side effects of our DNA vaccines were negligible in mice, thereby our research provides the basis foundations for clinical application. In fact, the FLK-1-based DNA vaccine is in its final preparation for Phase I clinical trials. Our research findings were also adapted by other investigators and extended to therapies of other diseases such as arteriosclerosis. Overall, research funded by this award provides for innovative novel strategies of immune therapies for both breast carcinoma and other diseases.

Appendix I: Publications

1. Niethammer,A.G., Xiang,R., Becker,J.C., Wodrich,H., Pertl,U., Karsten,G., Eliceiri,B.P., and Reisfeld,R.A. 2002. A DNA vaccine against VEGF receptor 2 prevents effective angiogenesis and inhibits tumor growth. *Nat.Med.* 8:1369-1375.
2. Luo,Y., Zhou,H., Mizutani,M., Mizutani,N., Reisfeld,R.A., and Xiang,R. 2003. Transcription factor Fos-related antigen 1 is an effective target for a breast cancer vaccine. *Proc.Natl.Acad.Sci.U.S.A* 100:8850-8855.
3. Xiang,R., Mizutani,N., Luo,Y., Chiodoni,C., Zhou,H., Mizutani,M., Ba,Y., Becker,J.C., and Reisfeld,R.A. 2005. A DNA vaccine targeting survivin combines apoptosis with suppression of angiogenesis in lung tumor eradication. *Cancer Res.* 65:553-561.
4. Zhou,H., Luo,Y., Mizutani,M., Mizutani,N., Reisfeld,R.A., and Xiang,R. 2005. T cell-mediated suppression of angiogenesis results in tumor protective immunity. *Blood* 106:2026-2032.
5. Zhou,H., Luo,Y., Lo,J.F., Kaplan,C.D., Mizutani,M., Mizutani,N., Lee,J.D., Primus,F.J., Becker,J.C., Xiang,R. *et al*. 2005. DNA-based vaccines activate innate and adaptive antitumor immunity by engaging the NKG2D receptor. *Proc.Natl.Acad.Sci.U.S.A* 102:10846-10851.
6. Zhou,H., Luo,Y., Kaplan,C.D., Kruger,J.A., Lee,S.H., Xiang,R., and Reisfeld,R.A. 2005. A DNA-based cancer vaccine enhances lymphocyte crosstalk by engaging the NKG2D receptor. *Blood*.
7. Reisfeld,R.A., Niethammer,A.G., Luo,Y., and Xiang,R. 2004. DNA vaccines designed to inhibit tumor growth by suppression of angiogenesis. *Int.Arch.Allergy Immunol.* 133:295-304.
8. Reisfeld,R.A., Niethammer,A.G., Luo,Y., and Xiang,R. 2004. DNA vaccines suppress tumor growth and metastases by the induction of anti-angiogenesis. *Immunol.Rev.* 199:181-190.
9. Mizutani,N., Luo,Y., Mizutani,M., Reisfeld,R.A., and Xiang,R. 2004. DNA vaccines suppress angiogenesis and protect against growth of breast cancer metastases. *Breast Dis.* 20:81-91.
10. Lee,S-H.*, Mizutani,N.*, Mizutani,M., Luo,Y., Zhou,H., Kaplan,C., Kim,S-W., Xiang,R., and Reisfeld,R.A. 2006. Endoglin (CD105) is a Target for an Oral DNA Vaccine against Breast Cancer. *Cancer Immunol. Immunotherapy* (in press)
11. Luo,Y., Zhou, H., Krueger,J., Kaplan,C., Dolman,C., Markowitz,D., Wu,W., Liu,L., Reisfeld,R.A., and Xiang,R. 2006. Targeting Tumor-Associated Macrophages: A Novel Strategy against Breast Cancer *J. Clin. Invest.* (revision)

A DNA vaccine against VEGF receptor 2 prevents effective angiogenesis and inhibits tumor growth

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Tumor cells are elusive targets for immunotherapy due to their heterogeneity and genetic instability. Here we describe a novel, oral DNA vaccine that targets stable, proliferating endothelial cells in the tumor vasculature rather than tumor cells. Targeting occurs through upregulated vascular-endothelial growth factor receptor 2 (FLK-1) of proliferating endothelial cells in the tumor vasculature. This vaccine effectively protected mice from lethal challenges with melanoma, colon carcinoma and lung carcinoma cells and reduced growth of established metastases in a therapeutic setting. CTL-mediated killing of endothelial cells indicated breaking of peripheral immune tolerance against this self antigen, resulting in markedly reduced dissemination of spontaneous and experimental pulmonary metastases. Angiogenesis in the tumor vasculature was suppressed without impairment of fertility, neuromuscular performance or hematopoiesis, albeit with a slight delay in wound healing. Our strategy circumvents problems in targeting of genetically unstable tumor cells. This approach may provide a new strategy for the rational design of cancer therapies.

The inhibition of tumor growth by attacking the tumor's vascular supply offers a primary target for anti-angiogenic intervention. This approach, pioneered by Folkman and colleagues[1–5], is attractive for several reasons. First, the inhibition of tumor-associated angiogenesis is a physiological host mechanism and should not lead to the development of resistance. Second, each tumor capillary has the potential to supply hundreds of tumor cells, so that targeting the tumor vasculature actually potentiates the antitumor effect. Third, direct contact of the vasculature with the circulation leads to efficient access of therapeutic agents[6].

Extensive studies by many investigators established that angiogenesis has a central role in the invasion, growth and metastasis of solid tumors[2,7–9]. In fact, angiogenesis is a rate-limiting step in the development of tumors since tumor growth is generally limited to 1–2 mm^3 in the absence of a blood supply[6,10]. Beyond this minimum size, tumors often become necrotic and apoptotic under such circumstances[11].

Because tumor cells frequently mutate in response to therapy and also downregulate major histocompatibility (MHC) antigens required for T cell–mediated antitumor responses[12,13], efforts have been made to eradicate tumors by therapies directed against the tumor microenvironment. One such report links calreticulin with a model viral tumor antigen, thus combining antitumor therapy with anti-angiogenesis[14]. Yet another approach is the administration of xenogeneic endothelial cells as a vaccine that yielded anti-angiogenic effects[15]. This approach differs from those of other investigators applying specific chemical or biological inhibitors of angiogenesis, which often require their constant administration at relatively high dose levels[16].

A more molecularly-defined alternative to xenoimmunization is offered by receptor tyrosine kinases (RTKs) and their growth-factor ligands required for tumor growth. Among these receptors, the vascular endothelial growth factor receptor 2 (VEGFR2, also known as FLK-1) that binds the five isomers of murine VEGF has a more restricted expression on endothelial cells and is upregulated once these cells proliferate during angiogenesis in the tumor vasculature. FLK-1 is strongly implicated as a therapeutic target, as it is necessary for tumor angiogenesis and has an important role in tumor growth, invasion and metastasis[7,8,17–24]. In fact, several approaches have been used to block FLK-1, including dominant-negative receptor mutants, germ-line disruption of VEGFR genes, monoclonal antibodies against VEGF and a series of synthetic RTK inhibitors[24,25].

Here we describe a novel strategy for achieving an antitumor immune response with a FLK-1-based DNA vaccine. Our vaccine causes the collapse of tumor vessels by evoking a T cell–mediated immune response against proliferating endothelial cells overexpressing this growth-factor receptor in the tumor vasculature.

A FLK-1 based DNA vaccine inhibits tumor growth

We tested our hypothesis by demonstrating that an effective antitumor immune response was induced against subcutaneous tumors by an orally administered DNA vaccine encoding murine FLK-1 carried by attenuated *Salmonella typhimurium*. To this end, we constructed the vector pcDNA3.1-FLK1 (Fig. 1*a*). Protein expression of FLK-1 was demonstrated by western blot-

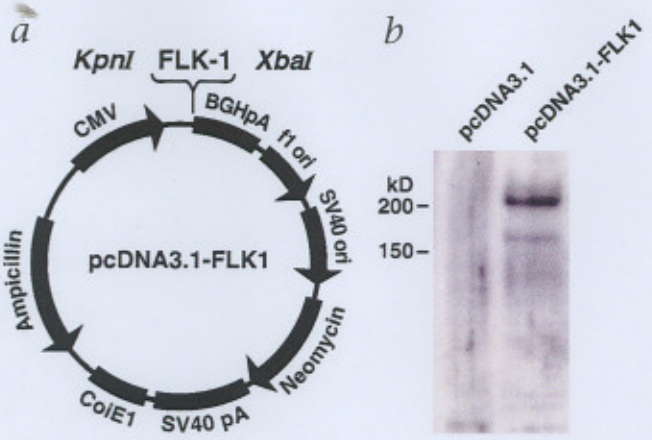

Fig. 1 Construction and functionality of expression vector. ***a***, The DNA encoding the entire murine Flk-1 gene was inserted into the pcDNA3.1 vector between the restriction sites *Kpn*I (5′) and *Xba*I (3′) ***b***, This construct was verified by nucleotide sequencing and protein expression by western blots after transient transfection into COS-7 cells. The protein appears in the lysate in its glycosylated form at 220 kD and to a lesser extent in its unglycosylated form at approximately 150 kD.

ting of transfected COS-7 cells (Fig. 1*b*). We established the efficacy of gene transfer from attenuated *S. typhimurium* into Peyer's patches by GFP expression in the cells derived from Peyer's patches at different time points after oral administration of mice (data not shown).

Marked inhibition of subcutaneous (s.c.) tumor growth was observed in C57BL/6J mice challenged two weeks after the third vaccination with pcDNA3.1-FLK1 by s.c. injection of either B16G3.26 murine melanoma cells or D121 non–small cell Lewis lung carcinoma cells (Figs. 2*a* and *b*). In contrast, animals vaccinated with only the empty vector pcDNA3.1, carried by the attenuated bacteria, revealed uniformly rapid s.c. tumor growth.

Prolonged antitumor effects were demonstrated since C57BL/6J mice challenged s.c. with MC-38 colon carcinoma cells 10 months after their last vaccination revealed a marked decrease in tumor growth in all experimental animals compared with controls (Fig. 2*c*).

Protection against spontaneous pulmonary metastases

We noted a marked reduction in dissemination of spontaneous pulmonary metastases in all experimental animals following three immunizations with the FLK-1-based DNA vaccine. This became evident 30 days after surgical excision of the s.c. primary Lewis lung carcinoma tumors, as confirmed by visual examination of the lungs of these animals, which established their metastatic score (Fig. 2*d*), as well as by histological analyses (data not shown).

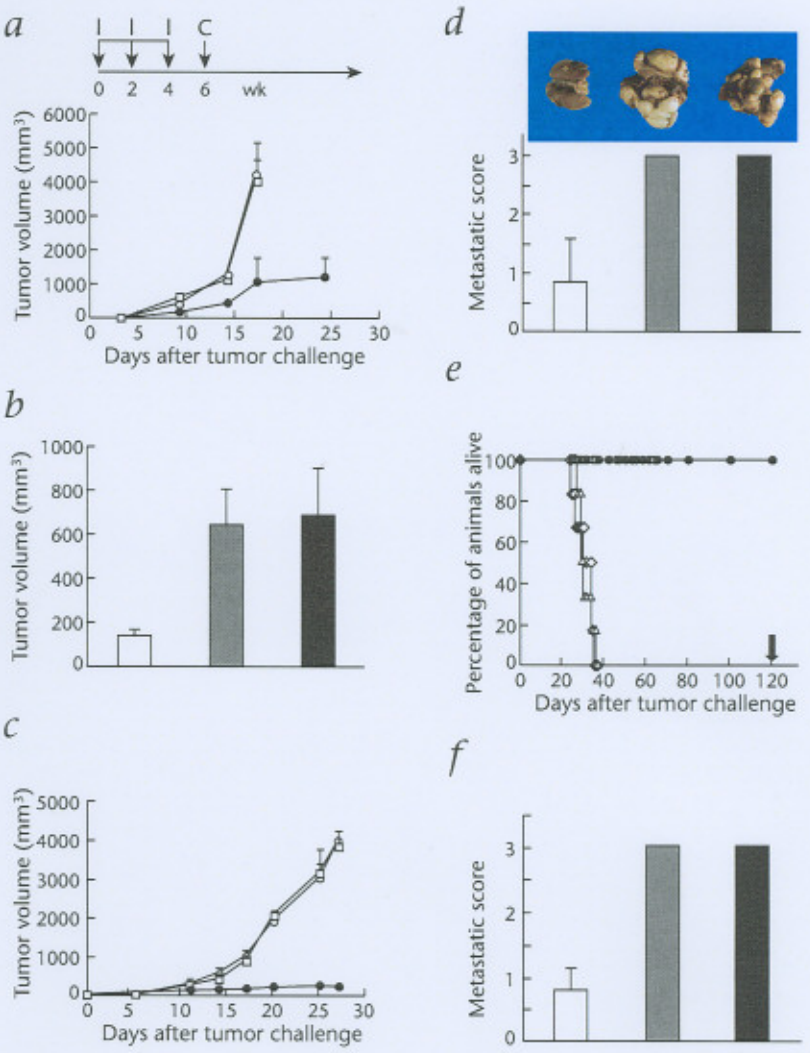

Fig. 2 Effect of the FLK-1 based DNA vaccine on tumor growth. ***a***, 2 wk after the last vaccination, mice were challenged (*c*). with a lethal dose of B16 melanoma cells. The average tumor growth of 8 mice is depicted. ●, immunization (I) with the vector encoding FLK-1; ○, control vector; □, PBS. $P < 0.01$. ***b***, C57BL/6J mice ($n = 8$) were challenged s.c. with a lethal dose of 1×10^5 D121lu Lewis lung carcinoma cells (□). Bar graphs indicate average tumor volume after 2 wk before tumor removal in comparison with mice that received only the control vector (▩) or PBS (■). Error bars indicate s.d. ***c***, Long-term effect of the pcDNA3.1-FLK1 vaccination was tested by challenging C57BL/6J mice ($n = 8$) with MC-38 colon carcinoma s.c. 10 months after the third immunization. Symbols are as in *e*. ***d***, Representative lung specimens of mice challenged with D121 Lewis lung carcinoma cells 4 weeks after removal of the subcutaneous primary tumors. Bars are as in *b*. ***e***, Lifespan of BALB/c mice after i.v. challenge with CT-26 colon carcinoma cells. The lifespan of groups of mice ($n = 6$) are shown following tumor cell challenge after repeated vaccinations. Death occurred in control groups due to extensive metastatic dissemination throughout the lung. 5 of the surviving mice were rechallenged (black arrow) to test for possible resistance. ●, vaccine; ◇, empty vector control; △, PBS. ***f***, Inhibition of tumor growth in a therapeutic setting. BALB/c mice ($n = 5$) were challenged i.v. with CT-26 colon carcinoma, and 10 d later 1 dose of the vaccine was applied. Experimental groups were scored 28 d after challenge. Bars are as in *b*.

Vaccination prolongs the lifespan of mice

We found a fourfold increase in lifespan of BALB/c mice ($n = 6$), vaccinated as described above, and challenged two weeks later by intravenous (i.v.) injection of a lethal dose of autologous CT-26 colon carcinoma cells (Fig. 2*e*). Possible resistance against our therapy was ruled out by rechallenging survivors ($n = 5$) 120 days after their first tumor-cell challenge and collecting their lungs 30 days later. Four mice did not reveal any tumors, whereas the one remaining animal had less then 10% of its lung surface covered by metastases (data not shown).

Vaccination reduces growth of established metastases

We established that our FLK-1-based DNA vaccine is also effective in a therapeutic setting. This was shown by i.v. injection of BALB/c mice ($n = 5$) with CT-26 colon carcinoma cells and vaccination of these mice 10 days thereafter with pcDNA3.1-FLK1 when they had fully established pulmonary metastases. All such treated mice survived and showed only few small lung foci, whereas all control animals treated with the empty vector or PBS began to die 28 days after tumor cell challenge (Fig. 2*f*).

$CD8^+$ T cells are responsible for the antitumor response

There was a marked increase in T-cell activation markers in splenocytes from successfully vaccinated C57BL/6J mice after a 12-hour incubation with B16G3.26 melanoma cells that had been stably transduced to express murine FLK-1. This included increased expression of CD25, the high-affinity interleukin-2 (IL-2)-receptor α chain, CD69 an early T-cell activation antigen and LFA-2 (CD2) a lymphocyte function-associated antigen (Fig. 3*a*). This upregulation was clearly evident when compared with $CD8^+$ T cells from mice vaccinated with pcDNA3.1-FLK1 but incubated with wild-type B16G3.26 melanoma cells ($P \leq 0.05$). Specific recognition of FLK-1 was indicated as no increase in expression was noted following co-incubation of cells expressing FLK-1 with splenocytes from C57BL/6J mice vaccinated with the empty vector. No such upregulation could be observed for $CD4^+$ T cells (data not shown).

We demonstrated the involvement of $CD8^+$ T cells in the antitumor immune response given that *in vivo* depletion of $CD8^+$ T cells—before i.v. challenge of vaccinated mice with CT-26 tumor cells—resulted in the complete abrogation or severe impairment of the antitumor response. In fact, mice depleted of $CD8^+$ T cells died within 45 days after tumor-cell challenge due to extensive growth and dissemination of pulmonary metastases (Fig. 3*b*). However, *in vivo* depletion of $CD4^+$ T cells did not decrease the effectiveness of our vaccine (data not shown).

Cytotoxic T cells associate with tumor endothelium

To reveal the localization of $CD8^+$ T cells to their target site, we stained these cells with fluorescein isothiocyanate (FITC) and

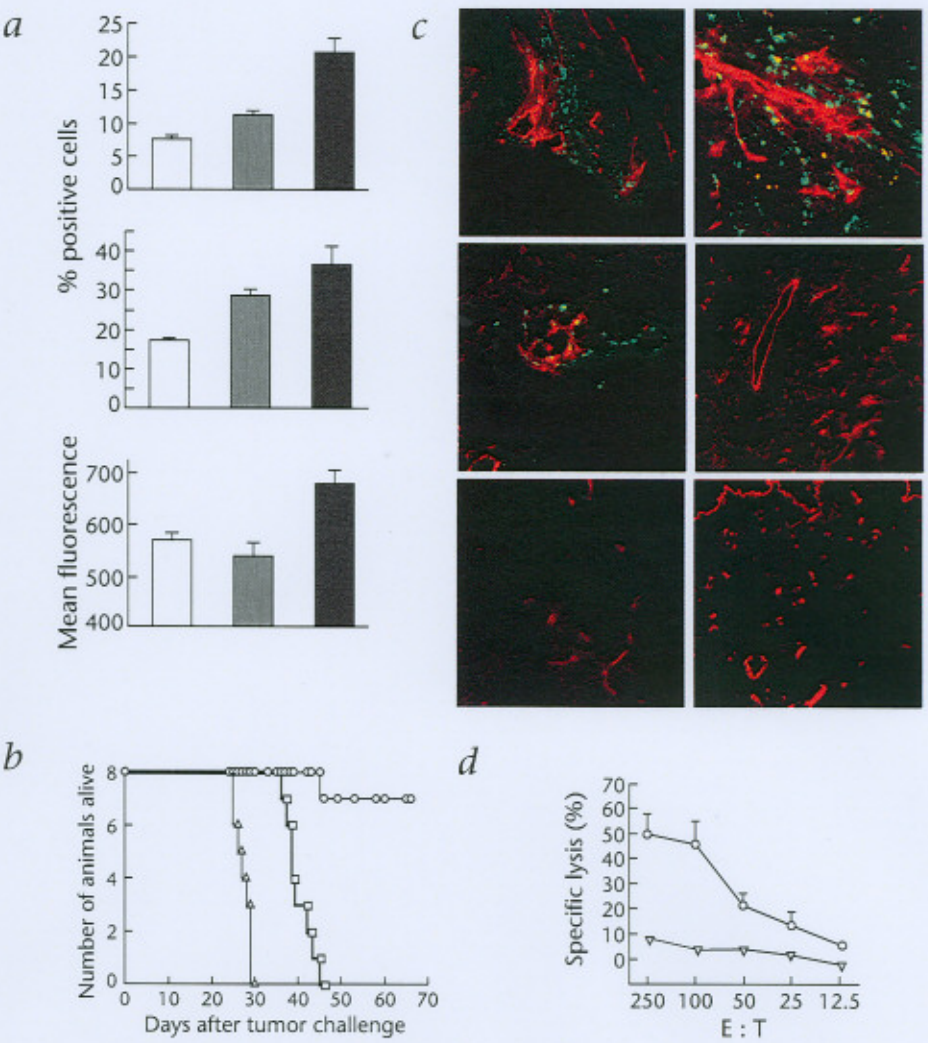

Fig. 3 Involvement of $CD8^+$ T cells. ***a***, Activation of $CD8^+$ T cells after *in vitro* co-incubation with cells expressing FLK-1. Shown is the increase in percentage of $CD8^+CD25^+$ (top) and $CD8^+CD69^+$ (middle) T cells, and increase in expression of CD2 on $CD8^+$ T cells (bottom) isolated from splenocytes of mice vaccinated with the vector pcDNA 3.1-FLK1 after cocultivation with cells expressing FLK-1 (■), or the identical cells lacking FLK-1 (■). A further control included $CD8^+$ T cells from mice immunized with the empty control vector and cocultivated with the B16 melanoma cell line expressing FLK-1 (□). Error bars indicate s.d. ***b***, Effect of $CD8^+$ T-cell depletion on lifespan. □, $CD8^+$ T-cell depletion during effector phase after vaccination with pcDNA3.1-FLK1; ○, no $CD8^+$ T-cell depletion of vaccinated mice; △, empty control vector. ***c***, Immunhistochemical analysis of $CD8^+$ T cells (FITC) and endothelial cells (rhodamine). Upper panels depict vascularized areas of CT-26 pulmonary metastases 4 mo after challenge and immunization with pc DNA3.1-FLK1. Left, ×20 magnification, 530 × 530 μm; right, ×40 magnification, 265 × 265 μm. Middle panels show Matrigel specimens after bFGF-induced vessel growth and prior immunizations with pcDNA3.1-FLK1 (left) and empty vector (right). Both panels, ×20 magnification. Lower panels reveal non-vascularized areas of tumor tissue (left) and adjacent skin tissue (right). Both panels, ×20 magnification. ***d***, Specific lysis of CT-26-FLK-1 cells by $CD8^+$ T cells from mice vaccinated with pcDNA3.1-FLK-1 (○) compared with $CD8^+$ T cells from control mice treated with empty vector (▽).

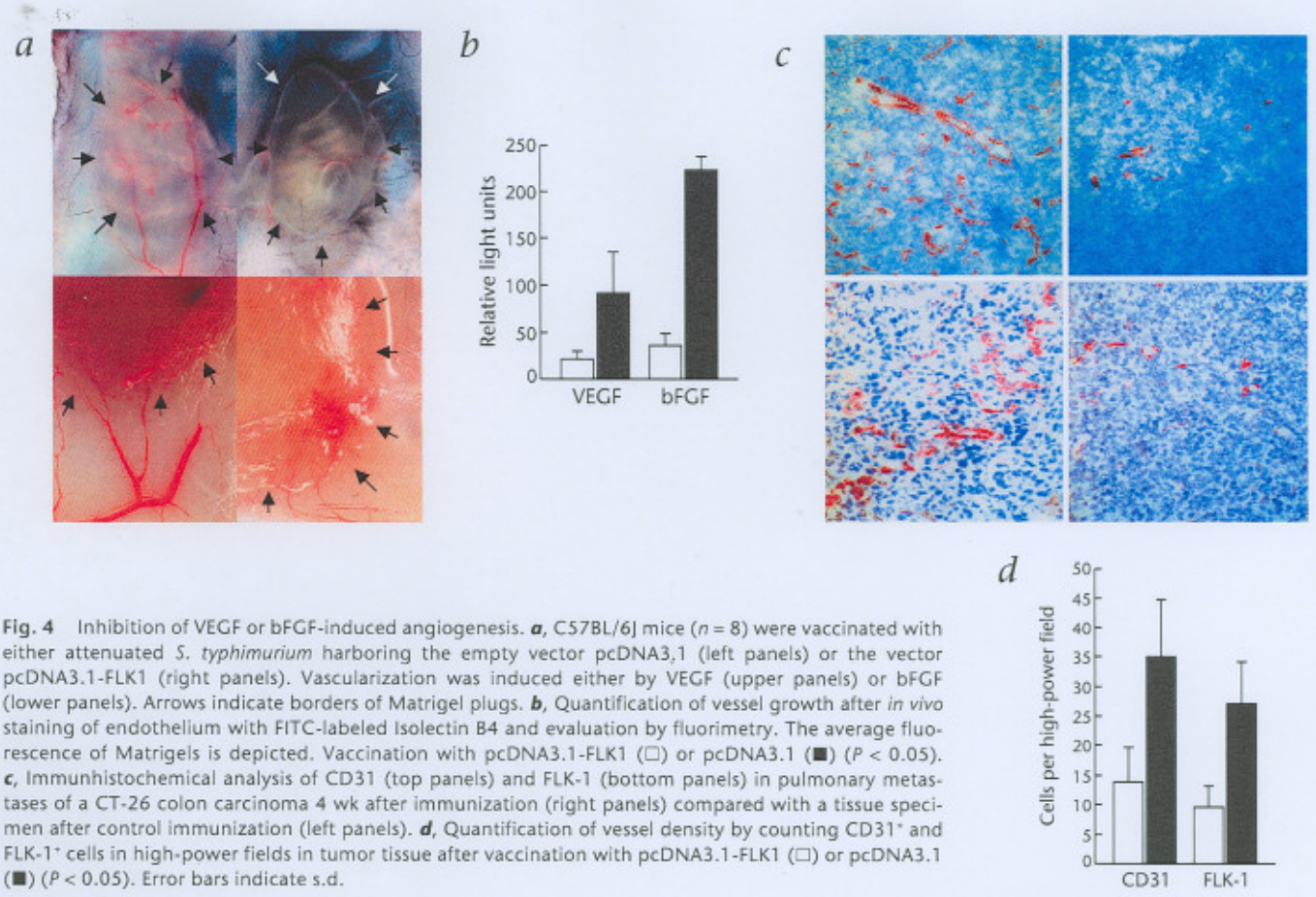

Fig. 4 Inhibition of VEGF or bFGF-induced angiogenesis. ***a***, C57BL/6J mice ($n = 8$) were vaccinated with either attenuated *S. typhimurium* harboring the empty vector pcDNA3,1 (left panels) or the vector pcDNA3.1-FLK1 (right panels). Vascularization was induced either by VEGF (upper panels) or bFGF (lower panels). Arrows indicate borders of Matrigel plugs. ***b***, Quantification of vessel growth after *in vivo* staining of endothelium with FITC-labeled Isolectin B4 and evaluation by fluorimetry. The average fluorescence of Matrigels is depicted. Vaccination with pcDNA3.1-FLK1 (□) or pcDNA3.1 (■) ($P < 0.05$). ***c***, Immunhistochemical analysis of CD31 (top panels) and FLK-1 (bottom panels) in pulmonary metastases of a CT-26 colon carcinoma 4 wk after immunization (right panels) compared with a tissue specimen after control immunization (left panels). ***d***, Quantification of vessel density by counting $CD31^+$ and $FLK\text{-}1^+$ cells in high-power fields in tumor tissue after vaccination with pcDNA3.1-FLK1 (□) or pcDNA3.1 (■) ($P < 0.05$). Error bars indicate s.d.

marked endothelial cells with rhodamine-conjugated antibody against CD31. Microscopic evaluation revealed an association of $CD8^+$ T cells with vessel structures throughout the tumor tissue or Matrigel sections of animals immunized with pcDNA3.1-FLK1. Almost no $CD8^+$ T cells were observed within non-vascularized, viable areas of tumor tissues even four months after tumor-cell challenge, nor were they associated with vessels in somatic tissues. Control vaccination did not induce any infiltration of cytotoxic T cells into tumor tissue or Matrigel (Fig. 3*c*).

Vaccination against FLK-1 induces T cell–mediated lysis

We demonstrated antigen-specific cytotoxicity against CT-26-FLK-1 cells with a standard 4-hour ^{51}Cr-release assay using splenocytes from BALB/c mice immunized against FLK-1 and challenged with CT-26 colon carcinoma cells. Immunizations with the vector encoding FLK-1 led to significant lysis of target cells by effector cells, in contrast to control immunizations (Fig. 3*d*). However, neither vaccination was effective in evoking any noticeable cytotoxicity against wild-type CT-26 cells not expressing FLK-1, thus excluding direct lysis of tumor cells (data not shown).

Reduction of neovascularization

We demonstrated distinct anti-angiogenic effects, independent of tumor cells, induced by the FLK-1-based DNA vaccine in a Matrigel assay. Differences were visible macroscopically, as shown in representative examples of Matrigel plugs removed six days after their installment (Fig. 4*a*). This was also evident from the extent of vascularization evaluated by relative fluorescence after *in vivo* staining of endothelium with FITC-conjugated lectin. There was a decrease in VEGF- or bFGF-induced neovascularization only after vaccination with the vector encoding FLK-1 but not with the empty vector (Fig. 4*b*). Immunohistochemical staining further revealed a decrease in vessel density among pulmonary metastases of CT-26 colon carcinoma after vaccination with pcDNA3.1-FLK1 as compared with tissue derived from control mice (Fig. 4*c*). Evaluation of high-power fields demonstrated decreased vessel density induced by the FLK-1-based vaccine (Fig. 4*d*).

Wound healing is delayed after vaccination

We noticed a measurable prolongation in the time required to completely close a total of 24 circular wounds inflicted on the backs of 6 mice immunized with the FLK-1-based vaccine versus that of mice immunized with the control vector (14.75 days, s.d. 1.5 versus 13.3 days, s.d. 1.6; $P<0.01$. This was accompanied by macroscopically visible swelling and inflammation in 11 of 24 versus 4 of 24 cases among controls (Fig. 5*a* and *b*).

Further experiments revealed no impact on fertility of mice based on the time elapsed from start of cohabitation until parturition nor on the number of pups born (Fig. 5*c* and *d*). All females of each experimental group gave birth. Neuromuscular performance as determined by both the wire test and footprint test, as well as by body weight, overall behavior and balancing tests, did not demonstrate any impairment attributed to vaccination (data not shown).

The occurrence of common, FLK-1-positive progenitor cells for both endothelial cells and hematopoietic cells led us to evaluate peripheral blood samples of C57BL/6J and BALB/c

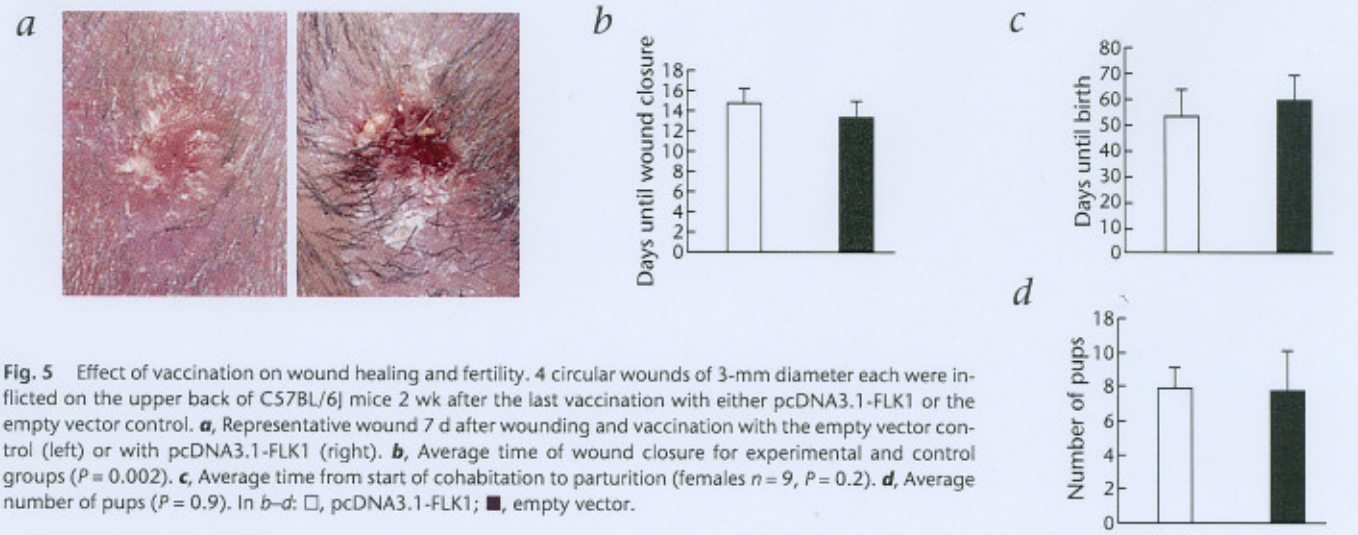

Fig. 5 Effect of vaccination on wound healing and fertility. 4 circular wounds of 3-mm diameter each were inflicted on the upper back of C57BL/6J mice 2 wk after the last vaccination with either pcDNA3.1-FLK1 or the empty vector control. ***a***, Representative wound 7 d after wounding and vaccination with the empty vector control (left) or with pcDNA3.1-FLK1 (right). ***b***, Average time of wound closure for experimental and control groups ($P = 0.002$). ***c***, Average time from start of cohabitation to parturition (females $n = 9$, $P = 0.2$). ***d***, Average number of pups ($P = 0.9$). In *b–d*: □, pcDNA3.1-FLK1; ■, empty vector.

mice up to ten months after their last immunization. However, total blood counts and differentials did not indicate any decreased or compensating hematopoiesis (data not shown).

Discussion

We developed a novel strategy that might overcome problems of tumor-cell heterogeneity and peripheral tolerance to self-antigens encountered in tumor cell–directed immunotherapy. We accomplished this by exploiting the obvious advantages of anti-angiogenic therapies developed by many other investigators[1–5]. In fact, there are several advantages of targeting $CD8^+$ T cells to proliferating endothelial cells in the tumor vasculature rather than directly to tumor cells. First, endothelial cells are genetically stable and do not downregulate MHC-class I and II antigens—an event that frequently occurs in solid tumors and severely impairs T cell–mediated antitumor responses[12]. In addition, immune suppression triggered by tumor cells at the cellular level can also be avoided by this approach. Second, the therapeutic target is tumor-independent, thus killing of proliferating endothelial cells in the tumor microenvironment can be effective against a variety of malignancies. Furthermore, proliferating endothelial cells are readily available to lymphocytes in the bloodstream. Consequently, the target tissue can be reached unimpaired by anatomical barriers such as the blood–brain barrier or encapsulation of tumor tissues[26].

Our studies show that peripheral T-cell tolerance against the murine VEGFR2 (FLK-1) can be broken by an oral DNA vaccine encoding autologous FLK-1, delivered by an attenuated strain of *S. typhimurium*. We induced antitumor immune responses in mouse tumor models of non–small cell lung carcinoma, colon carcinomas and melanoma, both against primary tumors and their respective spontaneous and experimental pulmonary metastases. Our DNA-vaccine was also effective in a therapeutic setting of established lung metastases. Additionally, we observed effective, prolonged antitumor effects evident after challenge up to ten months after the last immunization. Involvement of cytotoxic T cells in these events was suggested by marked upregulation of T-cell activation markers CD2, CD25, CD69 on $CD8^+$ T cells when co-incubated with cells expressing FLK-1. There was no apparent upregulation of these markers upon incubation with tumor cells. Furthermore, the effect of our vaccine was severely impaired in mice depleted *in vivo* of $CD8^+$ T cells throughout the effector phase. Depletion of $CD4^+$ cells was without effect. Importantly, angiogenesis was found to be effectively counteracted in a tumor cell–free, VEGF- or bFGF-induced Matrigel assay; both VEGF and bFGF upregulate the target antigen FLK-1 on endothelial cells[27]. Furthermore, *in vitro* cytotoxicity occurred only against target cells transduced to express FLK-1, but not against the identical wild-type cell line. We eliminated cytotoxicity mediated by $CD8^+$ T cells directly against tumor cells, as well as non-specific immune responses, as tumor protection was completely abrogated in mice that received attenuated *S. typhimurium* transformed with the empty vector lacking FLK-1.

We further demonstrated that fertility, neuromuscular performance and hematopoiesis of experimental mice remained unimpaired; anti-angiogenic effects induced by our DNA vaccine resulted in a slight but statistically significant delay in wound healing. However, the wounds of mice that were subjected to tumor excisions—including in some cases opening of the peritoneum—healed without any complications.

Together, our data show that the VEGFR2 is a suitable target for T cell–mediated immunotherapy within the tumor vasculature. Our findings may lead to a novel vaccine strategy for cancer therapy through the induction of an autoimmune response against self antigens expressed by proliferating endothelial cells. In fact, at least 46 transcripts are specifically elevated in the tumor-associated endothelium, thus providing a large array of potential candidates for this strategy[28]. This includes integrins or additional growth factor receptors and their ligands such as basic fibroblast growth factor or angiopoietin, as well as other molecules involved in their downstream signaling events[28]. It is also likely that DNA vaccines targeting proliferating endothelial cells could be used effectively in combination with specific inhibitors of angiogenesis, and chemotherapies or immunotherapies targeting the tumor cells themselves. Such combined approaches may ultimately lead to the rational design of novel and effective modalities for the treatment of cancer.

Methods

Animals, bacterial strains, and cell lines. Animal experiments were performed according to the National Institutes of Health *Guide for Care and Use of Experimental Animals* and approved by our Animal Care Committee (#ARC-43SEPT1). Attenuated *S. typhimurium* Aro/A (strain SL7207) was provided by B.A.D. Stocker. The D121 cell line was a gift from L. Eisenbach. Tumor tissues were screened for expression of FLK-1 by immunohistochemical staining and found to be negative; expression throughout the tumor-neovasculature was positive.

Construction of the expression vector encoding murine VEGFR-2 and transformation of *S. typhimurium*. DNA encoding murine VEGFR-2 (FLK-1) (provided by I. Lemischka) was cloned with the primers 3′-CCGGTA CCATGGAGAGCAAGGCGCTG-5′ and 5′-CCTCTAGACAGCAGCAC-CTCTCTC-3′ and inserted into the pcDNA3.1 vector (Invitrogen, San Diego, California) between the restriction sites *Kpn*1 and *Xba*1 generating pcDNA3.1-FLK1. Bacteria were electroporated as described[29,30].

Oral immunization and tumor-cell challenge. Mice were immunized by oral gavage 3 times at 2-wk intervals with 100 µl PBS containing 1×10^8 *S. typhimurium* transformed with pcDNA3.1-FLK1 or pcDNA3.1 as described[31]. C57BL/6J mice were challenged 2 wk later by s.c. injection of 1×10^5 B16G3.26 melanoma, MC-38 colon carcinoma or D121 lung carcinoma cells into the left front flank. Tumor volume was measured in 2 dimensions and calculated as follows: length/2 × width^2. Tumors of mice injected with D121 cells were excised after 2 wk to allow spontaneous dissemination to the lung. Metastatic scores were evaluated 4 wk later by the percentage of lung surface covered by fused metastases: 0% = 0; <20% = 1; 20–50% = 2; and >50% = 3. We injected CT-26 murine colon carcinoma cells (5×10^4) i.v. into BALB/c mice inducing experimental pulmonary metastases 2 wk after the last immunization. We tested our treatment in a therapeutic setting by vaccinating animals 10 d after i.v. injection of CT-26 cells.

Activation of CD8$^+$ T cells. We created the B16G3.26-FLK-1 melanoma and CT-26-FLK-1 colon carcinoma cell lines by retroviral transduction with FLK-1. One week after immunization, splenocytes were collected from C57BL/6J mice ($n = 4$), vaccinated with pcDNA3.1-FLK1 or the empty control vector. Cells were cocultured overnight with B16G3.26-FLK-1 or B16G3.26 tumor cells. Flow-cytometric analyses were performed using FITC-conjugated antibody to CD8 (#01044) in combination with PE-conjugated anti-mouse monoclonal antibodies to CD2 (#01175), CD25 (#01105A) or CD69 (#01505B) (BD-Pharmingen, La Jolla, California) as described[24]. We also used splenocytes in a standard 4-h ^{51}Cr-release assay to asses cytotoxicity against CT-26-FLK-1 and CT-26 target cells.

Evaluation of anti-angiogenic effects. C57BL/6J mice ($n = 8$) were injected into the sternal region with 250 µl growth factor–reduced Matrigel (#354230, BD Biosciences, Bedford, Massachusetts) containing 400 ng/ml murine VEGF (#450-32, PeproTech, Rocky Hill, New Jersey) or bFGF (#100-18B). Endothelium was stained 6 d later by i.v. injection of 200 µl (0.1 mg/ml) *Bandiera simplofica* lectin I, Isolectin B4 conjugated with fluorescein (Vector Labaratories, Burlingame, California). 30 min later, mice were killed and lectin-FITC was extracted from 100 µg per plug in 500 µl RIPA lysis buffer, centrifuged and its content in the supernatant quantified by fluorimetry (490 nm).

***In vivo* depletion of CD8$^+$ T cells.** We depleted CD8$^+$ T cells by weekly i.p. injections of 500 µg rat anti-mouse monoclonal antibody to CD8 (RH.495) as described[32]. Controls included non-depleted animals either vaccinated with pcDNA3.1-FLK1 or pcDNA3.1.

Immunohistochemistry. We stained cryosections (10 µm) fixed in paraformaldehyde. Antibody to CD31 (Pharmingen, San Diego, California) was incubated with rhodamine-conjugated secondary antibody, blocked with rat serum, followed by immunostaining with a FITC–conjugated antibody to CD8. Photomicrographs were captured with a laser scanning confocal microscope (Biorad, Hercules, California). Frozen tissue sections were stained with the Techmate Automate (Dako, Hamburg, Germany). Single stained serial sections were incubated for 30 min with biotinylated antibodies, followed by the streptavidin-peroxidase complex (DAKO) and the chromogen AEC (DAKO). Double stainings were performed as described[33]. Density of antigen-expressing cells was determined by counting of high-power fields.

Evaluation of possible side effects. To test wound healing, wounding was performed as described[34,35]. We inflicted 4 circular wounds of 3-mm diameter each on the upper back of C57BL/6J mice ($n = 6$), 2 wk after immunization with pcDNA3.1-FLK1 or the empty vector. Time until wound closure was noted. To evaluate fertility, 2 wk after the third immunization with either pcDNA3.1-FLK1 or with empty vector, female C57BL/6J mice ($n = 9$) were allowed to cohabitate with 3 males. The days until parturition and number of pups were noted. To test neuromuscular performance, we evaluated vaccinated and control mice by both the wire hang test and the footprint test[36,37] as well as by overall behavior and determination of body weight. To test hematopoiesis, animals were subjected to complete peripheral blood counts and differentials up to 10 mo after immunization.

Statistical analysis. The statistical significance of differential findings between experimental groups and controls was determined by Student's *t*-test and considered significant if two-tailed *P* values were <0.05.

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Competing interests statement

The authors declare that they have no competing financial interests.

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Transcription factor Fos-related antigen 1 is an effective target for a breast cancer vaccine

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Protection against breast cancer was achieved with a DNA vaccine against murine transcription factor Fos-related antigen 1, which is overexpressed in aggressively proliferating D2F2 murine breast carcinoma. Growth of primary s.c. tumor and dissemination of pulmonary metastases was markedly suppressed by this oral DNA vaccine, carried by attenuated *Salmonella typhimurium*, encoding murine Fos-related antigen 1, fused with mutant polyubiquitin, and cotransformed with secretory murine IL-18. The life span of 60% of vaccinated mice was tripled in the absence of detectable tumor growth after lethal tumor cell challenge. Immunological mechanisms involved activation of T, natural killer, and dendritic cells, as indicated by up-regulation of their activation markers and costimulatory molecules. Markedly increased specific target cell lysis was mediated by both MHC class I-restricted CD8$^+$ T cells and natural killer cells isolated from splenocytes of vaccinated mice, including a significant release of proinflammatory cytokines IFN-γ and IL-2. Importantly, fluorescence analysis of fibroblast growth factor 2 and tumor cell-induced vessel growth in Matrigel plugs demonstrated marked suppression of angiogenesis only in vaccinated animals. Taken together, this multifunctional DNA vaccine proved effective in protecting against growth and metastases of breast cancer by combining the action of immune effector cells with suppression of tumor angiogenesis.

vaccine | tumor | metastases | antiangiogenesis

Breast cancer, one of the most common malignancies in women, is second in cancer-causing death among women between the ages of 40 and 55 years in the United States (1). This neoplasm has been studied intensively, and recently new preventive measures and therapies emerged, including immunological and genetic treatments administered as adjuvant therapy after surgery, radiation, and chemotherapy. Biotherapy produced successful results in mice with mammary carcinoma, particularly with cellular vaccines (2), DNA vaccines (3, 4), recombinant proteins (5), and adoptive immunotherapy (6).

Progression of breast cancer often is accompanied by changes in gene expression patterns, resulting in highly tumorigenic and invasive cell types (7). In this regard, the transcription factor activating protein-1 (AP-1) family defines tumor progression and regulates breast cancer cell invasion and growth and resistance to antiestrogens (8). In addition, Fos-related antigen 1 (Fra-1), a transcription factor belonging to the AP-1 family, is overexpressed in many human and mouse epithelial carcinoma cells, including those of thyroid (9), kidney (10), esophagus (11), and breast (12). This overexpression greatly influences their morphology and motility, correlates with their transformation to a more invasive phenotype (13), and is specifically associated with highly invasive breast cancer cells (14). These findings suggest that overexpressed Fra-1 can serve as a potential target for active vaccination against breast cancer (15).

IL-18, a potent immunoregulatory cytokine, was described as an IFN-γ-inducing factor (16), which enhances cytokine production of T and/or natural killer (NK) cells and induces their proliferation and cytolytic activity (17). Tumor cells engineered to produce IL-18 are less tumorigenic (18), and systemic administration of IL-18 resulted in considerable therapeutic activity in several murine tumor models (19, 20). In addition, IL-18 enhances cellular immune mechanisms by up-regulating MHC class I antigen expression and favoring the differentiation of CD4$^+$ helper T cells toward the T helper 1 (Th1) subtype (21). In turn, Th1 cells secrete IL-2 and IFN-γ, which facilitate the proliferation and/or activation of CD8$^+$ cytotoxic T lymphocytes, NK cells, and macrophages, all of which can contribute to tumor regression (22). In addition, IL-18 is a novel inhibitor of angiogenesis, sufficiently potent to suppress tumor growth by directly inhibiting fibroblast growth factor 2-induced endothelial cell proliferation (23). Recently, the role of recombinant IL-18 as a biological "adjuvant" has been evaluated in murine tumor models, and its systemic administration induced significant antitumor effects in several tumor models (19, 24).

The induction of an efficacious Ag-specific immunity by DNA vaccines against self-Ag necessitates the optimization of vaccine design, including effective modalities of vaccine delivery, powerful adjuvants, and optimal antigen processing. This can be achieved by an oral vaccine delivery system using an attenuated strain of *Salmonella typhimurium* (*dam*$^-$ *aroA*$^-$) (25). Thus, vaccination by gavage of these bacteria harboring plasmid DNA vaccines proved effective for DNA delivery to such secondary lymphoid tissues as Peyer's patches in the small intestine, with subsequent induction of immunity against antigens encoded by the plasmid (26). DNA immunization also can be enhanced significantly by exploiting natural pathways of antigen presentation. Thus, most peptides presented as complexes with MHC class I antigens that induce active cytotoxic T lymphocytes are derived from cytosolic proteins degraded and processed by the proteasome. Protein is targeted to this organelle by polyubiquitination, a process in which many copies of this cellular protein are covalently attached to the target protein to markedly enhance its degradation by the proteasome (27).

Here, we describe a multifunctional DNA vaccine, encoding polyubiquitinated transcription factor Fra-1 and secretory IL-18, which effectively protects against primary breast tumor growth and metastases by suppression of tumor angiogenesis and activation of T, NK, and dendritic cells.

Materials and Methods

Animals, Bacterial Strains, and Cell Lines. Female BALB/c mice, 6–8 wk of age, were purchased from The Scripps Research Institute Rodent Breeding Facility. All animal experiments were performed according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The double attenuated *S. typhimurium* strain RE88 (*aroA*$^-$ *dam*$^-$) was kindly provided by the Remedyne Corporation (Santa Barbara, CA). The murine D2F2 breast cancer cell line was obtained from the American Type Culture Collection and cultured in DMEM supplemented with 10% (vol/vol) FBS.

Vector Construction and Protein Expression. Two constructs were made based on the pIRES vector (Invitrogen). The first, pUb-Fra-1, was comprised of polyubiquitinated, full-length murine Fra-1. The second, pIL-18, contained murine IL-18 with an Igκ leader sequence for secretion purposes. The empty vector with or

Abbreviations: Fra-1, Fos-related antigen 1; NK, natural killer; EGFP, enhanced GFP; PE, phycoerythrin; APC, antigen-presenting cell.

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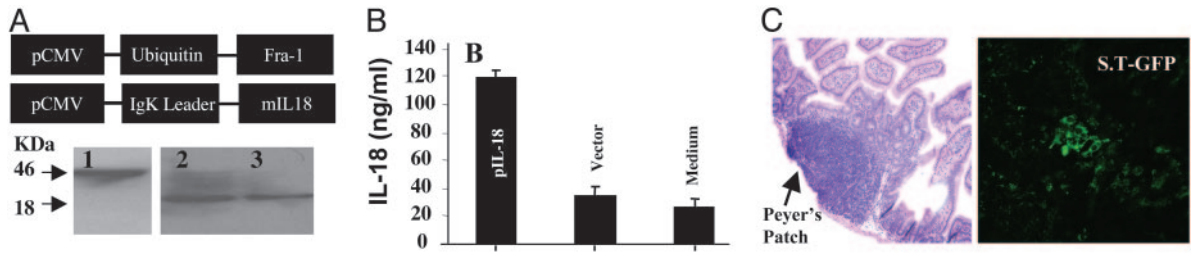

Fig. 1. Vector construction map, protein expression, bioactivity, and targeting of expression constructs. (*A*) The coding sequence of full-length, murine Fra-1, fused with polyubiquitin at the N terminus, was inserted into the pIRES plasmid (pUb-Fra-1). A second plasmid, pIL-18, contained the entire coding sequence of murine IL-18 with an Igκ leader sequence. Protein expression by pUb-Fra-1 and pIL-18 was demonstrated by Western blotting. Blots are shown at either pUb-Fra-1 (lane 1) or pIL-18 (lane 2) and of culture supernatant from pIL-18-transfected COS-7 cells (lane 3). (*B*) Bioactivity of IL-18 (ng/ml) determined by ELISA in supernatants of KG-1 lymphoma cells that transfected with pIL-18. The error bars indicate mean standard deviation of multiple assays. (*C*) Expression of EGFP activity in Peyer's patches was determined in mice immunized with 10^8 *aroA*$^-$ *dam*$^-$ bacteria transformed with pEGFP (S.T-GFP) by gavage. Fluorescence expression of EGFP was detected by confocol microscopy (*Right*). Hematoxylin/eosin staining of mouse Peyer's patches is shown (*Left*).

without ubiquitin served as a control. Protein expression of Fra-1 and IL-18 was demonstrated by Western blotting with a polyclonal rabbit anti-murine Fra-1 antibody (Santa Cruz Biotechnology) and a monoclonal anti-mouse IL-18 antibody (R & D Systems). Bioactivity of murine IL-18 in cell supernatants was measured by an ELISA (R & D Systems) using the production of IFN-γ in KG-1 lymphoma cells as an indicator (28).

Transformation and Expression of *S. typhimurium* with DNA Vaccine Plasmids. Attenuated *S. typhimurium* (*dam*$^-$, *aroA*$^-$) was transformed with DNA vaccine plasmids by electroporation. Freshly prepared bacteria (1×10^8) at midlog growth phase were mixed with plasmid DNA (2 μg) on ice in a 0.2-cm cuvette and electroporated at 2.5 KV, 25 μF, and 200 Ω. The bacteria were transformed with the following plasmids: empty vector, pUb, pUb-Fra-1, pIL-18, or both pUb-Fra-1 and pIL-18 combined, indicated as pUb-Fra-1/pIL-18. After electroporation, resistant colonies harboring the DNA vaccine gene(s) were cultured and stored at -70°C after confirmation of their coding sequence.

Detection of Enhanced GFP (EGFP) Expression. EGFP expression by *aroA*$^-$ *dam*$^-$ *S. typhimurium* was used to obtain direct evidence for DNA transfer from the bacterial carrier to Peyer's patches and to establish that protein expression took place efficiently and successfully. Mice were administered 1×10^8 bacteria by gavage and killed 24 h thereafter, and biopsies were collected from the small intestine. After washing, they were checked for EGFP expression in Peyer's patches by confocal microscopy or saved for further hematoxylin/eosin staining.

Immunization and Tumor Cell Challenge. Five groups of BALB/c mice ($n = 8$) were immunized three times at 2-wk intervals by gavage with 100 μl of PBS containing 1×10^8 doubly mutated *S. typhimurium* harboring either empty vector, pUb, pUb-Fra-1, pIL-18, or pUb-Fra-1/pIL-18. One week thereafter, mice were challenged either s.c. into the right flank with 1×10^6 D2F2 breast cancer cells or i.v. with 0.5×10^6 cells to induce primary tumor or experimental pulmonary metastases, respectively. The s.c. tumors were measured for width and length, and then volume was determined according to $(W^2 \times L)/2$. Pulmonary tumor metastases were examined and scored by a visual evaluation assessing the percentage of lung surface covered by fused metastases as follows: 0 = 0%, 1 = <20%, 2 = 20–50%, 3 = >50%.

Cytotoxicity Assay. Cytotoxicity was measured and calculated by a standard ^{51}Cr-release assay with splenocytes harvested 2 wk after challenge with 0.5×10^6 D2F2 cells, and subsequent culture for 3 d at 37°C in complete T-STIM culture medium (Becton Dickinson). Both ^{51}Cr-labeled D2F2 and Yac-1 cells were used as targets at various effector-to-target cell ratios.

Flow Cytometry. Activation markers of T and NK cells and CD80 and CD86 costimulatory molecules were measured by two-color flow cytometric analysis with a BD Biosciences FACScan. T cell activation was determined by staining freshly isolated splenocytes from successfully vaccinated mice with anti-CD8-FITC or anti-CD3-FITC Abs in combination with phycoerythrin (PE)-conjugated anti-CD25, CD11a, CD28, or CD69 Abs (PharMingen). Activation of NK cell markers was measured with FITC-labeled anti-NK-1.1 Abs in combination with PE-conjugated anti-DX5 Abs. Costimulatory molecules on antigen-presenting cells (APCs) were detected by PE-conjugated anti-CD80 or CD86 Abs in combination with FITC-labeled CD11c Abs.

Cytokine Release Assay. Flow cytometry was used for detection of intracellular cytokines and the ELISPOT assay to measure single-cell cytokine release. Splenocytes, collected 2 wk after D2F2 tumor cell challenge, were cultured for 24 h in complete T cell medium with irradiated D2F2 cells and assayed according to instructions provided by the manufacturer (BD Bioscience). Plates were read by IMMUNOSPOT SCANALYSIS, and digitalized images were analyzed for areas in which color density exceeded background by an amount based on a comparison with experimental wells.

Evaluation of Antiangiogenic Effects. Two weeks after the last vaccination, mice were injected s.c. in the sternal region with 500 μl of growth factor-reduced Matrigel (BD Biosciences) containing 400 ng/ml murine fibroblast growth factor 2 (PeproTech, Rocky Hill, NJ) and irradiated (1,000 Gy) D2F2 tumor cells (1×10^4/ml). In all mice, except for two controls, endothelium tissue was stained 6 d later by i.v. injection into the lateral tail vein of 200 μl of fluorescent *Bandeiraea simplicifolia* lectin I, Isolectin B4 at 0.1 mg/ml (Vector Laboratories). Thirty minutes later, Matrigel plugs were excised and evaluated macroscopically, and then Lectin-FITC was extracted by RIPA lysis buffer (0.15 mM NaCl/0.05 mM Tris·HCl, pH 7.2/1% Triton X-100/1% sodium deoxycholate/0.1% SDS) from 100-μg plugs to be quantified by fluorimetry at 490 nm. Background fluorescence found in the two noninjected control mice was subtracted in each case.

Results

Vectors Encoding Genes for Ub-Fra-1 or IL-18 Express the Respective Bioactive Protein. We successfully constructed the eukaryotic expression vectors based on the pIRES vector backbone, namely pUb-Fra-1 and pIL-18 (Fig. 1*A*). Protein expression of pUb-Fra-1 and pIL-18 was demonstrated by transient transfection of each vector into COS-7 cells and by performing Western blots of the

respective cell lysates (pUb-Fra-1 or pIL-18) and supernatants (pIL-18) with anti-Fra-1 and anti-IL-18 Ab, respectively. All constructs produced protein of the expected molecular mass with IL-18 being expressed in its active form at 18 kDa (Fig. 1*A*, lane 2) and Fra-1 as a 46-kDa protein (Fig. 1*A*, lane 1). Protein expression of IL-18 was also detected in the culture supernatant of transfected cells (Fig. 1*A*, lane 3). Importantly, the biofunctional activity of IL-18 was demonstrated by ELISA in supernatants of cells transfected with pIL-18 (Fig. 1*B*).

***S. typhimurium* Transfer Expression Vectors to Mouse Peyer's Patches.** DNA encoding pUb-Fra-1 and pIL-18 was successfully released from the attenuated bacteria and entered Peyer's patches in the small intestine (Fig. 1*C*). The DNA was subsequently transcribed by APCs, processed in the proteasome, and presented as MHC–peptide complexes to T cells. To this end, mice were administered by gavage 1×10^8 *dam*$^-$, *aroA*$^-$ attenuated *S. typhimurium*. After 24 h these animals were killed and biopsies were collected from the thoroughly washed small intestine. In fact, the doubly attenuated bacteria harboring EGFP (S.T-GFP) exhibited strong EGFP fluorescence (Fig. 1*C*). This finding suggested not only that such bacteria can transfer the target gene to Peyer's patches, but also that plasmids encoding each individual gene can successfully express their respective proteins. Importantly, these doubly attenuated bacteria do not survive very long because neither EGFP activity nor live bacteria could be detected in immunized animals after 72 h (data not shown). However, EGFP expression was detected in adherent cells, most likely APCs, such as dendritic cells and macrophages from Peyer's patches after oral administration of *S. typhimurium* harboring the eukaryotic EGFP expression plasmid. Taken together, these findings suggest that both plasmid transfer to and protein expression in eukaryotic cells did take place.

Tumor-Specific Protective Immunity Against Breast Cancer Is Induced by the DNA Vaccine. We proved our hypothesis that an orally administered DNA vaccine encoding murine Ub-Fra-1 and secretory IL-18, carried by attenuated *S. typhimurium*, can induce protective immunity against s.c. tumors and pulmonary metastases of D2F2 breast carcinoma. Thus, we observed marked inhibition of both s.c. tumor growth and disseminated experimental pulmonary metastases in BALB/c mice challenged 1 wk after the third vaccination with pUb-Fra-1/pIL-18 by either i.v. (Fig. 2*A*) or s.c. (Fig. 2*B*) injection of D2F2 murine breast cancer cells. In contrast, all animals vaccinated with only the empty vector (pIRES) or the vector encoding only ubiquitin (pUb), carried by attenuated bacteria, uniformly revealed rapid s.c tumor growth and extensive dissemination of pulmonary metastases. Importantly, the life span of 60% of successfully vaccinated BALB/c mice (5/8) was tripled in the absence of any detectable tumor growth up to 11 wk after tumor cell challenge (Fig. 2*C*).

MHC Class I-Restricted Tumor-Specific Cytotoxic T Lymphocytes and NK Cells Kill D2F2 Breast Cancer Cells *in Vitro*. Immunization with our DNA vaccine induces tumor-specific immunity capable of killing breast cancer cells *in vitro* either by MHC class I Ag-restricted CD8$^+$ T cells or NK cells. The data depicted in Fig. 3 indicate that CD8$^+$ T cells isolated from splenocytes of mice immunized with the vaccine encoding pUb-Fra-1/pIL-18 were effective in killing D2F2 breast cancer cells *in vitro* at different effector-to-target cell ratios. In contrast, controls such as CD8$^+$ T cells isolated from mice immunized with only the empty vector carried by attenuated *S. typhimurium* produced solely background levels of tumor cell lysis (Fig. 3). The CD8$^+$ T cell-mediated killing of D2F2 cells was specific because syngeneic prostate cancer target cells (RM-2) lacking Fra-1 were not lysed (data not shown). Importantly, the CD8$^+$ T cell-mediated tumor cell lysis was MHC class I Ag-restricted, because addition of 10 μg/ml anti-H-2K^d/H-2D^d Ab specifically inhibited lysis of D2F2 cells (Fig. 3).

A

Treatment groups	Metastatic Score
A. Empty vector	2, 3, 3, 3, 3, 3, 3, 3
B. pUb	3, 3, 3, 3, 3, 3, 3, 3
C. pUb-Fra-1	1, 1, 2, 2, 2, 2, 2, 3
D. pIL-18	0, 0, 1, 1, 1, 2, 2, 2
E. pUb-Fra-1/pIL-18	0, 0, 0, 0, 0, 1, 1, 2

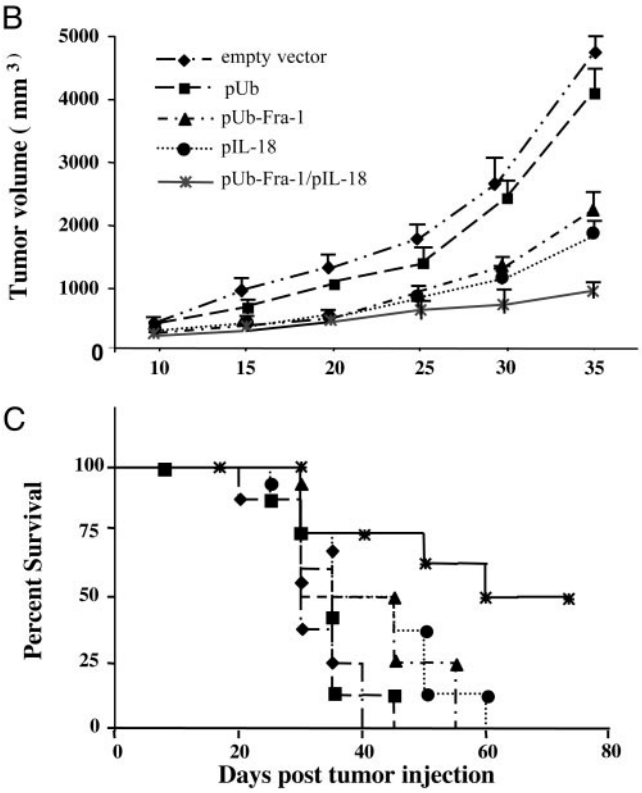

Fig. 2. Effect of the pUb-Fra-1/pIL-18-based DNA vaccine on primary tumor growth and metastases. Each experimental group ($n = 8$) of BALB/c mice was vaccinated by oral gavage as described in *Materials and Methods*. (*A*) Suppression of experimental pulmonary metastases of D2F2 breast carcinoma. Results are expressed as metastatic scores, i.e., percentage of lung surface covered by fused tumor foci. (*B*) Tumor growth was analyzed in mice challenged s.c. with 1×10^6 D2F2 tumor cells 1 wk after the last vaccination in each treatment or control group. (*C*) Survival curves represent results for eight mice in each of the treatment and control groups. Surviving mice were tumor free unless otherwise stated.

NK cells also were effective in tumor cell killing in a standard 4-h ^{51}Cr-release assay using NK-specific Yac-1 cells as targets for splenocytes from BALB/c mice immunized and challenged with D2F2 breast cancer cells. Lysis can also be achieved with breast cancer target cells, however, to a considerably lesser extent. Only immunization with the combined vectors, pUb-Fra-1/pIL-18, or pIL-18 alone led to significant lysis of Yac-1 target cells by NK cells, in contrast to control immunizations that were ineffective (Fig. 3).

Activation of T, NK, and Dendritic Cells. Interactions between IL-18 and active T helper 1 cells and NK cells are critical for achieving both optimal Ag-specific T cell and NK cell responses. The vaccine harboring either pUb-Fra-1/pIL-18 or pIL-18 alone up-regulated the expression of T or NK cell activation markers, respectively. This was evident from an increase in expression of CD25, the high-affinity IL-2R α-chain, CD69, an early T cell activation antigen, and CD11a, which is important for the initial interaction between T cells

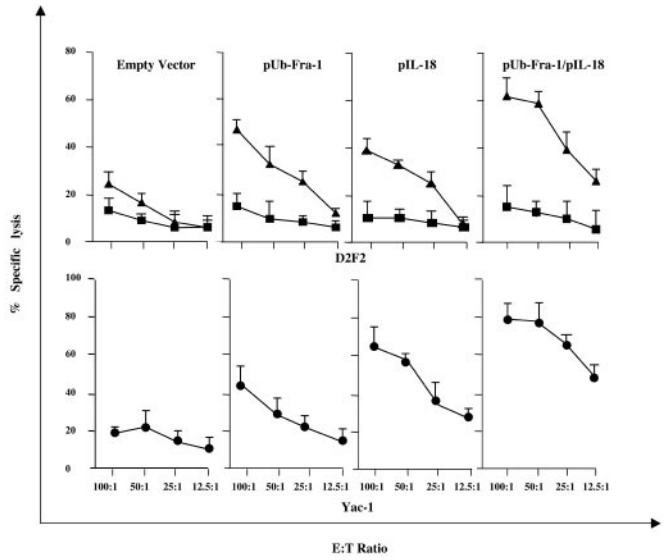

Fig. 3. Cytotoxicity induced by $CD8^+$ T and NK cells. Splenocytes were isolated from BALB/c mice after vaccination with experimental or control DNA vaccines 2 wk after challenge with D2F2 tumor cells and analyzed for their cytotoxic activity in a ^{51}Cr-release assay at different effector-to-target cell ratios. (*Upper*) Specific lysis mediated by $CD8^+$ T cells against D2F2 target cells (▲), which was blocked by an anti-MHC-class I Ab ($H\text{-}2K^d/H\text{-}2D^d$) (■). (*Lower*) Lysis mediated by NK cells (●) against Yac-1 target cells. Each value shown represents the mean of eight animals.

and dendritic cells, and regular T cell markers $CD4^+$ and $CD8^+$ (Fig. 4). Additionally, it is known that NK cells can play a partial role in antitumor immune responses (29). For this reason, spleen cells obtained from mice successfully immunized with DNA vaccines along with the control groups were assayed with anti-DX5 antibody (Fig. 5), demonstrating that this regimen dramatically increased DX5 expression on NK cells, which is especially important for NK cytotoxity.

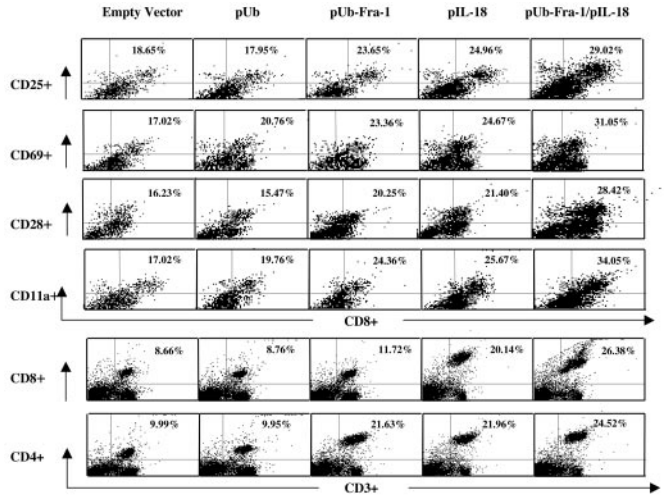

Fig. 4. Up-regulated expression of T cell activation molecules. Two-color flow cytometric analyses were performed with single-cell suspensions of splenocytes obtained from immunized mice. Anti-CD25, CD69, CD28, and CD11a Ab were used in PE-conjugated form in combination with FITC-conjugated anti-mouse mAb directed against $CD8^+$ T cells. PE-labeled anti-CD8 and anti-CD4 Abs were used in combination with FITC-conjugated anti-mouse mAb CD3. Each value represents the mean for four mice. Differences between the two control groups (empty vector and Ub) were statistically significant when compared with the three treatment groups ($P < 0.05$).

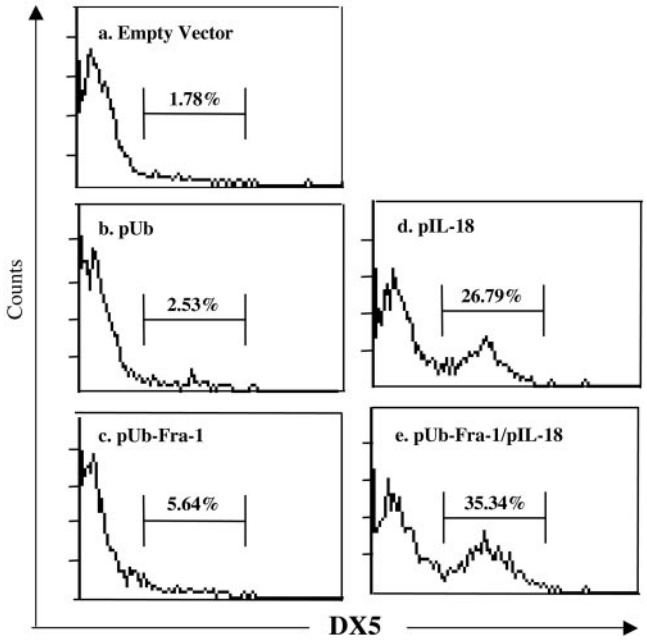

Fig. 5. Increased expression of NK cell marker after administration of DNA vaccine. Fluorescence-activated cell sorting analysis of splenocytes with anti-DX5 mAb revealed the activation of NK cells after DNA vaccination. The experimental setting is similar to that of Fig. 4. Percentages refer to cells gated scored positive for DX5 expression. A representative histogram plot is shown for each group with the value depicting the mean for four mice. Differences between the two control groups (*a* and *b*) and two treatment groups (*d* and *e*) were statistically significant ($P < 0.05$).

Furthermore, T cell activation is known to critically depend on up-regulated expression of costimulatory molecules CD80 and CD86 on dendritic cells to achieve optimal ligation with CD28 expressed on activated T cells. In this regard, fluorescence-activated cell sorting analyses of splenocytes obtained from syngeneic BALB/c mice, successfully immunized with the DNA vaccine, indeed demonstrated that we accomplished this particular task successfully, as the expression of CD80 and CD86 was up-regulated 2- to 3-fold over controls (Fig. 6).

T Cell Activation Is Indicated by Increased Secretion of Proinflammatory Cytokines. The release of proinflammatory cytokines IL-2 and IFN-γ from T cells is a well-known indication of T cell activation in

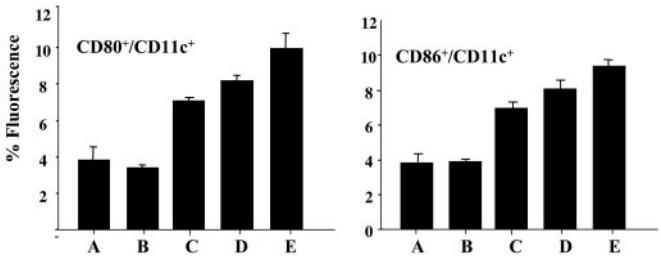

Fig. 6. Up-regulation of costimulatory molecules by DNA vaccine. In the same experiment as that depicted in Fig. 4, two-color flow cytometric analyses were performed with single-cell suspensions prepared from mouse splenocytes obtained 30 d after tumor cell challenge. Splenocytes were stained with FITC-labeled anti-CD11c Ab in combination with PE-conjugated anti-CD80 or CD86 Abs. Shown is the percent fluorescence of cell surface expressions of these two costimulatory molecules in each group ($n = 4$) (mean + SD). Differences between the two control groups (A and B) and the three treatment groups (C–E) were statistically significant ($P < 0.05$).

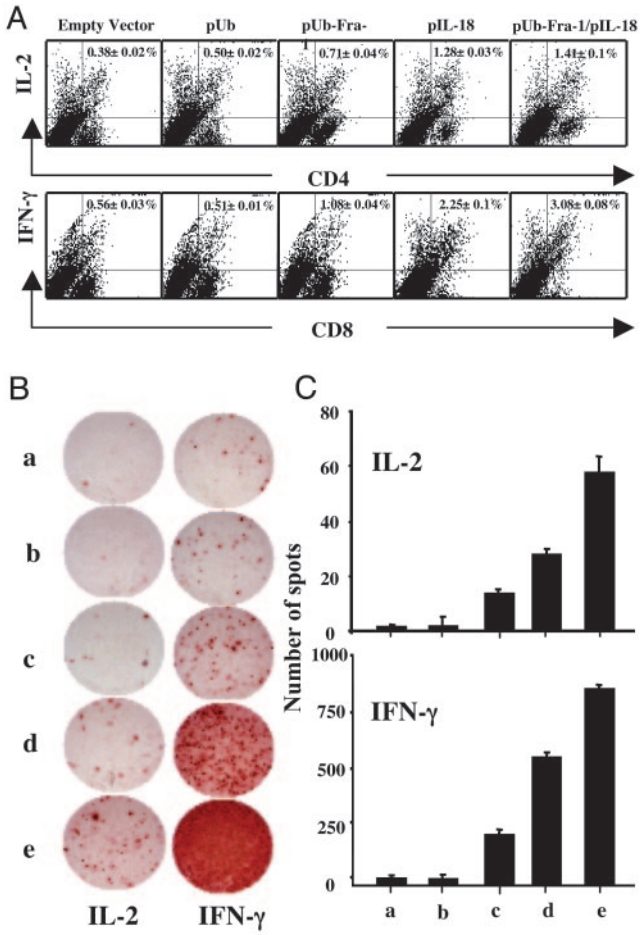

Fig. 7. Induction of cytokines at the intracellular and single T cell level. (*A*) Cytokines at the intracellular level were determined in splenocytes obtained 2 wk after tumor cell challenge and stained with FITC-anti-CD4 or CD8 Abs. Cells were fixed, permeabilized, and subsequently stained with PE-labeled anti-IFN-γ or anti-IL-2 Abs to detect the intracellular expression of these cytokines. Representative dot plots are shown for each group with the value depicting the mean for eight mice. (*B*) Production of IFN-γ and IL-2 was verified at the single-cell level by measuring reproduction in individual T cells by the ELISPOT assay. A representative ELISPOT assay is shown as spot formation per well induced by empty vector (*a*), pUb (*b*), pUb-Fra-1 (*c*), pIL-18 (*d*), and pUb-Fra-1/pIL-18 (*e*). (*C*) The mean spot distribution of each well in each experimental and control group is shown ($n = 4$, mean ± SD). Differences between the two control groups (a and b) and the three treatment groups (c–e) are statistically significant with treatment group (e) being most significant ($P < 0.001$).

secondary lymphoid tissues. When we measured these two cytokines, both intracellularly with flow cytometry (Fig. 7*A*) and at the single-cell level by ELISPOT (Fig. 7 *B* and *C*), we found that vaccination with the pUb-Fra-1/pIL-18 plasmid and subsequent challenge with tumor cells induced a dramatic increase in IFN-γ and IL-2 release over that of splenocytes from controls.

Suppression of Angiogenesis Is Induced by DNA Vaccine. We could demonstrate distinct suppression of angiogenesis induced by the pUb-Fra-1/pIL-18 DNA vaccine in a Matrigel assay. This was evident from the marked decrease in vascularization after vaccination, as evaluated by relative fluorescence after *in vivo* staining of endothelium with FITC-conjugated lectin. Differences were visible macroscopically, as shown in Fig. 8, depicting representative examples of Matrigel plugs removed from vaccinated mice 6 d after their injection. FITC-lectin staining clearly revealed suppression of angiogenesis, as indicated by a significantly decreased vascularization in Matrigel plugs only after vaccination with the vector encoding pUb-Fra-1/pIL-18 and to a somewhat lesser extent with pIL-18 alone, but not with vaccines encoding only pUb-Fra-1, pUb, or the empty vector control (Fig. 8).

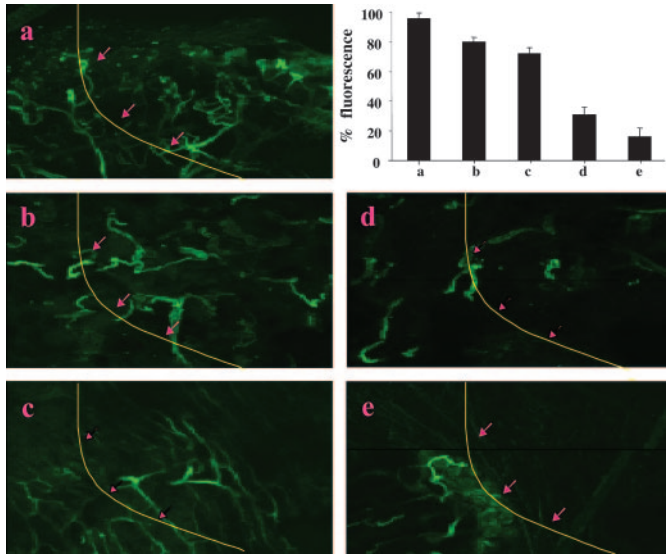

Fig. 8. Suppression of tumor angiogenesis. Antiangiogenesis was determined by the Matrigel assay. Quantification of vessel growth and staining of endothelium was determined by fluorimetry or confocal microscopy, respectively, using FITC-labeled Isolectin B4. The line and arrows (*a*–*e*) indicate the inside borders of the Matrigel plug. Matrigel was implanted into mice vaccinated with empty vector (*a*), pUb (*b*), pUb-Fra-1 (*c*), pIL-18 (*d*), or pUb-Fra-1/pIL-18 (*e*). The average fluorescence of Matrigel plugs from each group of mice is depicted by the bar graphs ($n = 4$; mean + SD). Comparison of control groups (*a* and *b*) with treatment groups (*d* and *e*) indicated statistical significance ($P < 0.05$). (Magnification: ×200.)

Discussion

Our data suggest that peripheral T cell tolerance against the Fra-1 transcription factor was broken by our DNA vaccine, fused with mutant polyubiquitin, and modified by cotransformation with a gene encoding secretory murine IL-18. This resulted in a prominent cellular immune response by $CD4^+$ T cells, $CD8^+$ T cells, and NK cells, tightly controlled by up-regulation of IFN-γ (29). Secretion of IFN-γ is further restricted by the availability of IFN-γ-inducing cytokines such as IL-2, IL-12, and tumor necrosis factor α, which are secreted from accessory cells after activation. IL-18 is another IFN-γ regulator (17), reported also to be a potent antiangiogenic cytokine, both *in vitro* and *in vivo* (30). Our vaccine design was successful because activation of both T and NK cells was significantly augmented. In fact, $CD8^+$ T cell activation was indicated by critically dependent up-regulation of costimulatory molecules CD80 and CD86 on APCs, resulting in optimal ligation with CD28 on activated T cells. Indeed, our data indicate up-regulation of these costimulatory molecules on $CD11c^+$ and MHC class II Ag-positive APCs, suggesting that their capability for presentation of tumor-specific Ag was significantly enhanced.

The marked increase in proinflammatory cytokines IFN-γ and IL-2 also demonstrated T cell activation as did the up-regulation of CD25, especially because it occurred together with increased production of IL-2 by activated T cells. Importantly, tumor angiogenesis was effectively suppressed only in mice immunized with pUb-Fra-1/pIL-18 and to a lesser extent with pIL-18 alone as indicated by suppression of vessel formation and regression of growing blood vessels.

Our success in eliciting an effective $CD8^+$ T cell-mediated MHC class I Ag-restricted tumor protective immunity with a completely

autologous oral DNA vaccine was most likely aided by our efforts to optimize antigen processing in the proteasome with polyubiquitination (31, 32). Support for this contention comes from our findings indicating that a DNA vaccine encoding murine Fra-1 lacking in ubiquitin was considerably less effective in inducing tumor protective immunity (data not shown). In contrast, the polyubiquitinated DNA vaccine was clearly capable of inducing tumor-protective immunity against a lethal challenge of D2F2 breast cancer cells.

One of the more critical aspects of DNA vaccine design is the selection of an optimally effective carrier to deliver the target gene to secondary lymphoid organs, such as Peyer's patches, in the small intestine. Live, attenuated bacterial carriers that harbor eukaryotic expression plasmids encoding Ag, combined with powerful adjuvants, are attractive vehicles for oral delivery of vaccines. Current DNA vaccine delivery vehicles include nonreplicating attenuated strains of intracellular bacteria like *S. typhimurium*, *Listeria monocytogenes*, and *Mycobacterium bovis* as well as *Bacillus Calmette Guerin*. These DNA vaccine delivery vehicles were reported to induce a broad spectrum of both mucosal and systemic immune responses. Moreover, the use of this natural route of entry could prove to be of benefit because many bacteria, like *Salmonell*a, egress from the gut lumen via the M cells into Peyer's patches (26) and migrate eventually into lymph nodes and spleen, thus allowing natural targeting of DNA vaccines to inductive sites of the immune system.

The doubly mutated strain of *S. typhimurium* (dam^-, $aroA^-$) was used as a delivery vehicle for our DNA vaccine because it was shown to be highly attenuated and useful as a live vaccine in a murine model of infection (33). Additionally, this mutant does not cause a transient state of nonspecific immune suppression and thus is useful for delivering heterologous antigens to immune inductive sites (34). Although dam^- mutants were found to be unable to cause disease in mice, transient bacteria remained after several weeks in terminal organs (35). Thus, to completely abolish the systemic presence of the bacteria, a second mutation ($aroA^-$) was introduced that inhibits the synthesis of aromatic amino acids and causes the bacteria to die after just a few passages.

We not only demonstrated that the Fra-1 antigen targets appropriate pathways of MHC class I Ag processing and presentation, but also that an adequate cytokine milieu is generated that effectively promotes Ag-specific responses. The most prominent advantage of this vaccine carrier vehicle is its ability to directly target DNA vaccines intralymphatically to Peyer's patches, which harbor immature dendritic cells, B cells, T cells, and macrophages, i.e., most of the important effector cells necessary for an immune response induced by a DNA vaccine. In fact, Maloy *et al.* (36) clearly demonstrated that intralymphatic immunization enhances DNA vaccination, increasing immunogenicity by 100- to 1,000-fold while inducing strong and biologically relevant $CD8^+$ cytotoxic T lymphocyte responses.

Taken together, our studies demonstrate that the transcription factor Fra-1 is a suitable target for induction of a T cell-mediated specific immune response against D2F2 breast cancer cells, and that the design of a DNA vaccine, especially polyubiquitination of the encoded protein immunogen and utilization of an attenuated bacterial carrier, lead to effective Ag processing and presentation, which result in effective tumor-protective immunity. The coexpression of secretory IL-18 by our vaccine acts as a powerful and natural adjuvant for further activation of both $CD8^+$ and $CD4^+$ T cells and NK cells, leading to the production of IFN-γ and IL-2 and importantly, to the suppression of effective angiogenesis in the tumor vasculature. It is anticipated that this multifunctional DNA vaccine may aid in the rational design of such vaccines for the immunotherapy of human breast cancer.

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A DNA Vaccine Targeting Survivin Combines Apoptosis with Suppression of Angiogenesis in Lung Tumor Eradication

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Abstract

A novel strategy achieved the eradication of lung tumor metastases by joint suppression of angiogenesis in the tumor neovasculature and induction of tumor cell apoptosis. This was accomplished by CTLs induced by a DNA vaccine encoding secretory chemokine CCL21 and the inhibitor of apoptosis protein survivin, overexpressed by both proliferating endothelial cells in the tumor vasculature and tumor cells. Oral delivery of this DNA vaccine by doubly attenuated *Salmonella typhimurium* (dam$^-$ and AroA$^-$) to such secondary lymphoid organs as Peyer's patches in the small intestine, elicited marked activation of antigen-presenting dendritic cells, and an effective CD8$^+$ T cell immune response against the survivin self-antigen. This resulted in eradication or suppression of pulmonary metastases of non–small cell lung carcinoma in both prophylactic and therapeutic settings in C57BL/6J mice. Moreover, the suppression of angiogenesis induced by the vaccine did not impair wound healing or fertility of treated mice. It is anticipated that such novel DNA vaccines will aid in the rational design of future strategies for the prevention and treatment of cancer. (Cancer Res 2005; 65(2): 553-61)

Introduction

The effective suppression and treatment of metastatic non–small cell lung carcinoma remains a major challenge for oncology since clinical outcome is relatively poor. In fact, cancer immunotherapies designed for direct attacks on tumor cells face longstanding limitations, including poor immunogenicity of tumor self-antigens and the genetic instability of tumor cells which often combine to make tumor cells the most difficult and elusive targets for current immunotherapies (1–4).

Recently, genetic immunizations with DNA-based vaccines provided a promising new approach for cancer immunotherapy (5–13), which offers several advantages over conventional vaccinations. First, sequence motifs, such as unmethylated CpGs of some bacterial plasmids are immunostimulatory and can function as vaccine adjuvants. Second, coexpression of appropriate chemokines or cytokines by such vaccines can enhance their efficacy and generate an effective immune response. Third, DNA-based vaccines can be highly effective by evoking a long-lived memory, T cell–mediated, tumor-protective immune response. Despite all these attractive features, current DNA-based cancer vaccines still require constant improvements, primarily by selection of the most efficacious target antigen(s), effective molecular adjuvants, as well as suitable carriers that will trigger robust, multiple cell-mediated antitumor effects against different compartments of the tumor microenvironment.

The pioneering efforts of Dario Altieri and other investigators provided an extensive amount of information on the structure and function of the inhibitor of apoptosis protein survivin, particularly its role in apoptosis and cell cycle regulation of cancer cells (14–23). In view of this large body of information, the small, 16.5-kDa inhibitor of apoptosis protein survivin is an almost ideal target for a DNA-based cancer vaccine (24). Thus, because survivin is overexpressed by essentially all solid tumors, it lends itself to broad therapeutic applications, especially because it is poorly, if at all expressed by normal adult cells and tissues. In fact, in gene-profiling studies, survivin was identified as the fourth "TRANSCRIPTOME" expressed in the most common human cancers but not in normal tissues (24). Survivin also was shown to be essential for cancer cell viability because it is one of the most important factors that regulate the balance between cell proliferation and programmed cell death during the cell cycle (15, 18, 24). In fact, survivin is highly regulated and optimally expressed in the G_2-M phase of the cell cycle, and its interaction with the mitotic spindle apparatus is essential for its antiapoptotic function (15, 17, 19). However, the complexity of the survivin pathway may extend beyond tumor cell populations, particularly because its increased expression by proliferating endothelial cells during the proliferative and remodeling phases of angiogenesis generates a cytoprotective mechanism for these cells (25, 26). Based on these facts, an attractive alternative to a direct attack solely on tumor cells will be to inhibit tumor growth and metastasis by simultaneously attacking both the tumor and its vasculature via an effective CTL response against survivin and thereby triggering tumor cell apoptosis and suppression of angiogenesis. This combined immunologic attack further extends the antiangiogenic intervention strategy pioneered by Folkman (27, 28) and offers an additional strategy for cancer therapy.

To further enhance the efficacy of our survivin-based cancer vaccine and, in particular, to overcome the poor immunogenicity of this tumor self-antigen, we coexpressed the multifunctional murine chemokine CCL21 since it binds to CXCR3 in addition to its own CCR7 receptor (29, 30) and effectively chemoattracts activated antigen-presenting dendritic cells (DCs) and naive T cells, bringing them together in lymphoid follicles and secondary lymphoid organs such as Peyer's patches for an effective T cell–mediated immune response (31, 32). Because CCL21 also binds to CXCR3 in the mouse, similar to the two angiostatic chemokines CXCL9 and CXCL10, its antitumor activity may be boosted in part by its angiostatic activity (33). This was shown by intratumoral (33), s.c. (34), or intralymphoid (35) injection of CCL21, which inhibited growth and metastasis of human tumor xenografts in SCID mice associated with a reduction in vascularity.

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Polyubiquitination has been used for several of our DNA vaccines, particularly since we found in one of our initial studies that the presence of mutant polyubiquitin upstream of a DNA minigene encoding melanoma peptide antigens proved to be essential for achieving tumor-protective immunity (36). Based on the well-known role of polyubiquitin in protein processing by the proteasome, we assumed that this molecule was essential for optimizing antigen processing and ultimately effective antigen presentation in the MHC class I antigen pathway (36).

Here, we show for the first time the antitumor efficacy of a novel strategy employing two distinct but complementary pathways, one of apoptosis induction and the other of angiogenesis suppression, both triggered by CTLs against survivin evoked by an oral survivin-based DNA vaccine coexpressing chemokine CCL21.

Materials and Methods

Animals, Bacterial Strains, and Cell Lines. Female C57BL/6J mice, ages 6 to 8 weeks, were purchased from The Scripps Research Institute's Rodent Breeding Facility and maintained at our animal facility. All animal experiments were done in compliance with the NIH Guides for the Care and Use of Laboratory Animals and approved by the Animal Care Committee of The Scripps Research Institute. The attenuated *Salmonella typhimurium* strain RE88 (dam^- and $AroA^-$) was provided by Remedyne Corp., (Santa Barbara, CA). Bacterial strain DH5α was purchased from Invitrogen, (Carlsbad, CA) and bacteria were routinely grown at 37°C in LB broth or on agar plates (EM SCIENCE, Darmstadt, Germany), supplemented, when required, with 25 μg/mL zeocin. The murine D121 lung cancer cell line was a gift from Dr. L. Eisenbach (Rehovoth, Israel).

Gene Cloning and Construction of Expression Vectors for DNA Vaccination. The full-length coding regions for murine survivin and CCL21 were amplified by reverse transcription-PCR of total RNA (1 μg) extracted from D121 mouse Lewis lung carcinoma cells and activated mouse splenocytes, respectively. Total RNA was extracted with the RNeasy Mini kit (Qiagen, Valencia, CA) and reverse transcription-PCR was done with a platinum quantitative reverse transcription-PCR thermoscript one-step system (Invitrogen) according to the manufacturer's instructions. Several constructs were made based on the pBudCE4.1 vector (Invitrogen) by using the PCR products, designed for independent expression of two genes from a single plasmid in mammalian expression vectors. The first of these constructs, pBud-CCL21 contains the *CCL21* gene, which is inserted into the multiple cloning site A under the control of the P_{CMV} promoter. The second construct, pBud-survivin, contains mutant ubiquitin, inserted into the multiple cloning site B site under control of the $P_{EF\text{-}1\alpha}$ promoter, with the survivin gene being fused to the COOH-terminal of ubiquitin thus forming the polyubiquitinated protein. The last construct, pBud-survivin/CCL21, expresses both genes independently. The empty vector was generated as a control. These constructs are illustrated schematically in Fig. 1*A*. The sequences and protein expressions of all constructs were shown by sequence analysis and Western blotting (Fig. 1*B*).

Oral Vaccination and Tumor Challenge. C57BL/6J mice were divided into five groups for each experiment and were immunized thrice at 2-week intervals by gavage with 100 μL PBS, containing 1×10^8 colony-forming unit's attenuated *S. typhimurium* (RE88), harboring one of the following: empty vector pBud; individual expression vectors of either pBud-survivin/CCL21, pBud-survivin, or pBud-CCL21 along with a PBS control group. All mice used in the prophylactic experiments were challenged by i.v. injections of 1×10^5 D121 murine Lewis lung carcinoma cells 1 week after the last immunization. In therapeutic settings, mice were first injected i.v. with 1×10^5 D121 murine Lewis lung carcinoma cells and 5 days later subjected to three vaccinations, 5 days apart. Mice were examined daily, sacrificed, and examined for lung metastasis 28 days after tumor cell challenge in the prophylactic setting or 25 days after the initial tumor cell inoculation in the therapeutic model.

Determination of Antiangiogenic Effects. Mice were vaccinated as described above. Two weeks after the last vaccination, mice were injected s.c. in the sternal region with 500 μL growth factor-reduced Matrigel (BD Biosciences, La Jolla, CA.) containing 400 ng/mL murine fibroblast growth factor-2 (PeproTech, Rocky Hill, NJ) and D121 tumor cells (1×10^4/mL) which were irradiated with 1,000 Gy. In all mice, except for two control animals, endothelium tissue was stained 6 days later by injecting into the lateral tail vein 200 μL of 0.1 mg/mL fluorescent *Bandeiraea simplicifolia* lectin I, Isolectin B4 (Vector Laboratories, Burlingame, CA); 30 minutes later, mice were sacrificed and Matrigel plugs excised and evaluated macroscopically. Lectin-FITC was then extracted from 100 μL of each plug with 500 μL of radioimmunoprecipitation assay buffer lysis buffer and then quantified by fluorimetry at 490 nm. Background fluorescence found in the two non-injected control mice was subtracted in each case.

Cytotoxicity Assay. Splenocytes were isolated from vaccinated mice 5 days after tumor cell challenge. Cytotoxicity was assessed by a standard ^{51}Cr release assay (36) against targets of either D121 tumor cells or murine endothelial cells overexpressing survivin. To determine specific MHC class I restriction of cytotoxicity, the inhibition experiments were done with 10 μg/mL anti-mouse MHC class I $H\text{-}2K^b/D^b$ monoclonal antibody (mAb; BD PharMingen, San Diego, CA).

Flow Cytometric Analysis. Activation markers of T cells and expression of costimulatory molecules on CD11c and MHC class II Ag-positive DCs were determined by two- or three-color flow cytometric analyses with a BD Biosciences FACScan. T cell activation was evaluated by staining freshly isolated splenocytes from vaccinated mice with FITC-labeled anti-CD3e mAb in combination with PE-conjugated anti CD25, CD28, or CD69 mAbs. Activation of costimulatory molecules on APCs was measured with FITC-labeled anti-CD11c mAb and biotinylated anti-IA^b mAb, followed by streptavidin-allophycocyanin, and in combination with PE-conjugated anti-ICAM-1, CD80, or DEC205 mAbs. All reagents for these assays were obtained from BD PharMingen.

Cytokine Release Assay. Flow cytometry was used for detection of intracellular cytokines. To this end, splenocytes were collected from C57BL/6J mice 2 weeks after D121 tumor cell challenge and cultured 24 hours in complete T cell medium together with irradiated D121 cells as described previously (36). Preincubated cells were suspended with 1 μg purified 2.4G2 mAb to block nonspecific staining. The cells were washed and then stained with 0.5 μg FITC conjugated anti-CD4 or anti-CD8 mAbs. After washing twice, cells were fixed and stained with 1 μg/mL PE conjugated with either anti-IL2 or anti-IFN-γ mAbs for flow cytometric analysis. All mAbs were obtained from BD PharMingen.

Analysis of Tumor Cell Apoptosis. Apoptosis in D121 tumor cells induced by vaccination was measured at 3 and 24 hours, respectively. Both control and experimental animals were challenged i.v. with 1×10^5 D121 cells 1 week after the last of three immunizations. Splenocytes were harvested from each individual mouse 1 week after tumor cell challenge and thereafter 2.5×10^7 splenocytes were cocultured for 4 hours with 5×10^5 D121 tumor cells in 6-well plates. Adherent tumor cells were easily separated from splenocytes in suspension. The ANNEXIN V-FITC apoptosis detection kit II (BD Biosciences) was used for confirmation of early stage of apoptosis. To confirm later stage tumor cell apoptosis, 5×10^5 D121 cells and 2.5×10^7 splenocytes were cocultured for 24 hours and isolated tumor cells were then analyzed by fluorescence-activated cell sorting for apoptosis by the TUNEL assay with the APO-DIRECT KIT (BD Biosciences) according to the manufacturer's instructions.

Evaluation of Possible Side Effects. Wound healing was tested by wounding as described (37, 38). Wounds of 3-mm lengths were inflicted on the upper backs of C57BL/6J mice ($n = 4$), 1 week after three immunization of each of the experimental groups. The time until wound closure was noted. To evaluate fertility, 1 week after the third immunization with either the survivin/CCL21 vaccine or PBS, female C57BL/6J mice ($n = 6$) were allowed to cohabitate with males, in a 3:1 breeding ratio. The days until parturition and number of pups were noted.

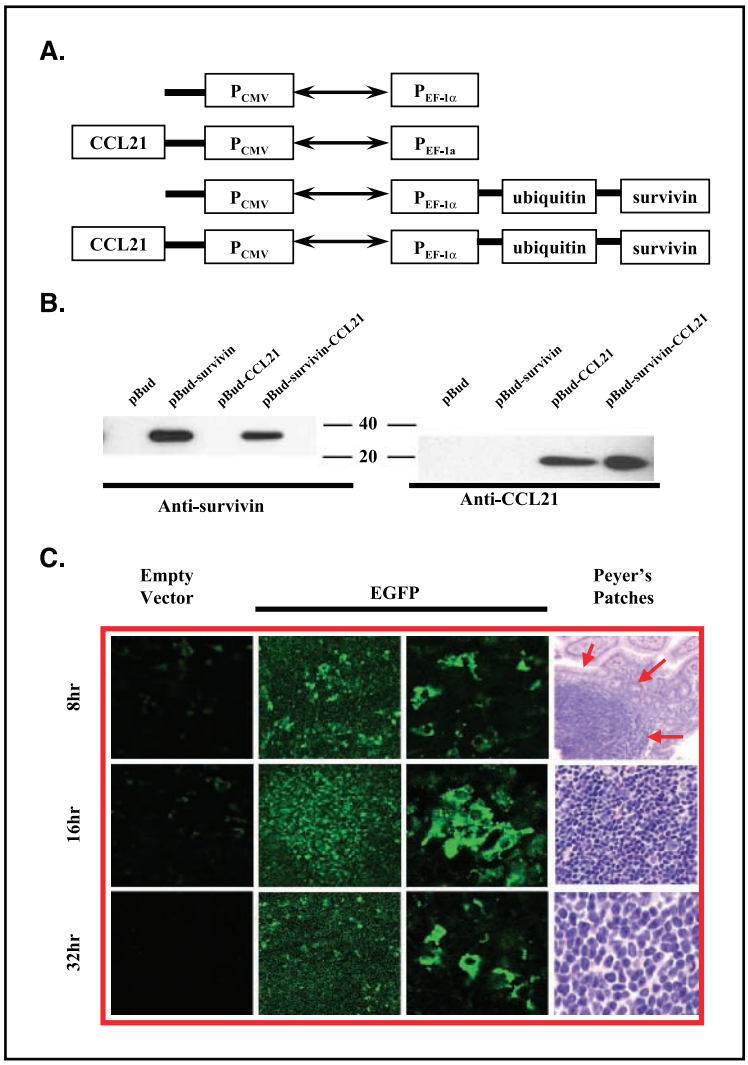

Figure 1. Construction and functional assay of expression vectors encoding ubiquitin, survivin, and CCL21. *A,* three expression vectors were constructed encoding either ubiquitin with survivin, CCL21, or both based on the pBudCE4.1 plasmid backbone. *B,* protein expressions of survivin and CCL21 were detected by Western blotting of cell lysates following transfection of plasmids into COS-7 cells using antisurvivin and anti-CCL21 Abs, respectively. *C,* expression of EGFP activity in Peyer's patches of C57BL/6J mice was detected in mice after oral administration of 10^8 CFU *S. typhimurium* (dam$^-$ and AroA$^-$) transformed with pEGFP. Mice were sacrificed at time points of 8, 16, and 32 hours and fresh specimens of small intestine were removed for analysis after thoroughly washing with PBS. Fluorescence expression of EGFP was detected by confocol microscopy (*left*). H&E staining of mouse Peyer's patches (*red arrows*; *right*) for analysis of biopsies taken from mouse small intestine.

Statistical Analysis. The statistical significance of differential findings between experimental groups and controls was determined by Student's *t* test and considered significant if two-tailed $P < 0.05$.

Results

A Survivin-Based DNA Vaccine Is Delivered to Peyer's Patches. We tested the hypothesis that a DNA vaccine encoding the inhibitor of apoptosis protein survivin, overexpressed in both tumor cells and proliferating endothelial cells in the tumor vasculature, induces a T cell–mediated immune response that triggered both tumor cell apoptosis and suppression of angiogenesis which led to the eradication of lung tumor metastases. This vaccine, which also coexpressed the secretory chemokine CCL21, was delivered orally by attenuated *Salmonella typhimurium* (dam$^-$ and AroA$^-$). To this end, three eukaryotic expression vectors were constructed based on the pBudCE4.1 vector backbone encoding either CCL21, survivin, or both survivin/CCL21, using either the P_{CMV} and/or $P_{EF\text{-}1\alpha}$ promoter (Fig. 1*A*). Protein expression of CCL21 and survivin was shown by transient transfection of each vector into COS-7 cells and by performing Western blots on the respective cell lysates with anti-CCL21 or antisurvivin mAbs indicating that the constructs produced proteins of the expected molecular mass (Fig. 1*B*).

We previously reported the effective *in vitro* DNA transfer from single mutant *S. typhimurium* (AroA$^-$), harboring the EGFP expression vector, to mouse primary peritoneal macrophages (36). Here, the new RE88 strain of doubly mutated *S. typhimurium* (dam$^-$ and AroA$^-$) was shown to successfully deliver plasmids to mouse Peyer's patches *in vivo* (Fig. 1*C*). Such plasmids still expressed the relevant protein as determined by immunization of mice by gavage with doubly attenuated *S. typhimurium*, carrying the EGFP mammalian expression vector as a reporter gene. When such animals were sacrificed and the Peyer's patches collected from the thoroughly washed small intestines, a strong fluorescence was observed by confocal microscopy (Fig. 1*C*). These data suggest that this doubly attenuated strain of *Salmonella typhimurium* not only transfers the respective plasmid to Peyer's patches, but also that such plasmids encoding the target gene can still express the respective protein *in vivo*.

Vaccination Protects Against Pulmonary Tumor Growth and Metastases. We showed that the survivin-based vaccine can induce effective suppression of tumor growth and metastases by evoking an effective T cell–mediated immune response, which triggers both tumor cell apoptosis and suppression of tumor angiogenesis. In fact, in a prophylactic setting, eradication, or suppression of

disseminated pulmonary metastases of D121 murine Lewis lung carcinoma were observed in mice vaccinated thrice at 2-week intervals and then challenged 1 week later by i.v. injection of tumor cells. Indeed, 6 of 8 mice completely rejected all pulmonary tumor metastases, whereas the remaining two animals revealed markedly reduced tumor metastases (Fig. 2*A*). In contrast, the survivin-based DNA vaccine lacking CCL21 was far less effective and induced complete suppression of metastases in only 1 of 8 animals, whereas all remaining mice showed extensive metastatic tumor growth. Additional animals that were treated only with control vaccinations of either PBS or empty vector revealed no tumor protection at all and died within 4 weeks after tumor cell challenge due to extensive metastases. Although immunization with doubly attenuated *Salmonella* carrying only the secretory CCL21 plasmid did not dramatically suppress tumor metastasis, it still resulted in statistically significant delays of metastases when compared with controls (Fig. 2*A*).

Vaccination Reduces Growth of Established Metastases. The survivin- or CCL21-based DNA vaccine proved also to be effective in markedly suppressing the growth of already well established pulmonary metastases in all experimental animals in a therapeutic setting (Fig. 2*B*). In contrast, all mice receiving only the survivin- or CCL21-based vaccines per se, or empty vector and PBS controls, revealed disseminated pulmonary metastases of D121 non–small cell lung carcinoma in this experimental setting (Fig. 2*B*).

CTL-Mediated Apoptosis Is Induced by Survivin Targeting. A critical question was answered when we found that the antitumor immunity observed was induced by the triggering of CTL-mediated tumor cell apoptosis. In fact, results of two key experiments indicated that incubation of D121 tumor cells with splenocytes from mice successfully vaccinated, with the survivin/CCL21 vaccine resulted in tumor cell apoptosis. Early apoptosis was detected at 3 hours by the ANNEXIN V assay and with a considerable further increase after 24 hours by the TUNEL assay as indicated by flow cytometric analyses (Fig. 3*A*). Thus, early stage apoptosis was up to 3- to 4-fold higher in groups of mice immunized with the survivin/CCL21 vaccine than in controls after splenocytes harvested from such mice were coincubated with tumor cells. Adherent tumor cells were easily separated from splenocytes in suspension. Importantly, a dramatic 85% increase in apoptosis was observed at 24 hours (Fig. 3*B*) only in mice immunized with the survivin/CCL21 vaccine, suggesting that the robust tumor cell immunity triggered this event.

A marked CTL response was induced by the survivin/CCL21 vaccine indicated by specific *in vitro* lysis of tumor cells which was mediated only by splenocytes isolated from such immunized mice. In fact, a standard ^{51}Cr release assay revealed marked cytotoxicity induced by specific CD8$^+$ T cells obtained from mice after vaccination and subsequent challenge with D121 Lewis lung carcinoma cells (Fig. 3*C*). In contrast, CD8$^+$ T cells isolated from control animals were found to be completely ineffective in evoking any noticeable killing of tumor cells as they evoked only background cytotoxic activities (Fig. 3*C*). Characteristically, the T cell–mediated cytotoxicity observed was MHC class 1 antigen restricted because it was completely abolished by the addition of anti-H2K^b/H2D^b mAbs (Fig. 3*C*). Taken together, these results suggest that CTL-mediated lysis plays a significant role in the increased apoptosis of D121 tumor cells.

Vaccination Induces Suppression of Angiogenesis in the Tumor Neovasculature. A key question of our study was answered when we found that the survivin/CCL21–based vaccine,

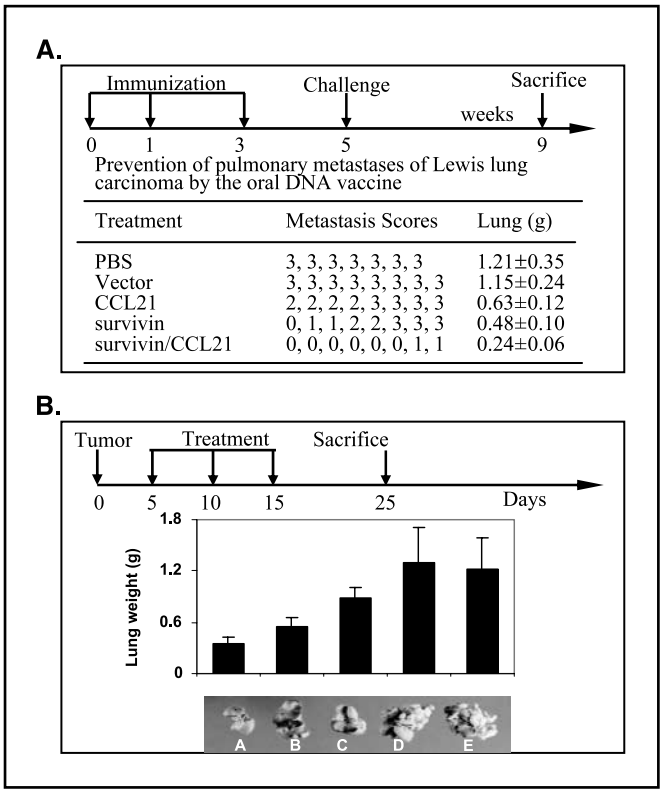

Figure 2. Suppression of pulmonary metastases of Lewis lung carcinoma by the oral DNA vaccines in both prophylactic and therapeutic treatment models. Lung metastases were induced in the prophylactic setting by i.v. injection of 1×10^5 D121 cells 1 week after the last of three immunizations administered by gavage at 2-week intervals. Experiments were terminated 28 days after tumor cell inoculation and the extent of pulmonary tumor foci determined. *A,* tumor metastasis scores and lung weights following immunization with either PBS, empty vector, CCL21, survivin, or survivin/CCL21 vaccines, respectively. Results: metastasis scores expressed as the % lung surface covered by fused metastatic foci: 0, none; 1, <5%; 2, 5% to 50%; and 3, >50%. Differences in metastasis scores and lung weights between groups of mice treated with the survivin/CCL21 vaccine and all control groups were statistically significant ($P < 0.001$). *B,* inhibition of tumor growth in the therapeutic model. C57BL/6J mice were first injected i.v. with 1×10^5 D121 Lewis lung carcinoma cells and received 5 days later DNA vaccines by gavage as indicated. Lung weights of the various experimental groups and pictures of lung specimens of each group. (*A-E*) survivin/CCL21, survivin, CCL21, empty vector, and PBS. Normal lung weight = 0.2 g.

which was already shown to be capable of triggering the induction of tumor cell apoptosis (Fig. 3*A* and *B*), also decisively suppressed angiogenesis in the tumor vasculature. This was shown by a significant decrease in tumor neovascularization indicated by Matrigel assays and their quantification by relative fluorescence measured after *in vivo* staining of mouse endothelium with FITC-conjugated lectin (Fig. 4*A*). These data were further corroborated by the detection of macroscopically evident differences among experimental groups and control groups of mice upon examination of representative Matrigel plugs removed 6 days after s.c. injection, following an i.v. injection of FITC-conjugated lectin (Fig. 4*B*). These experiments were repeated thrice with essentially the same results.

A possible mechanism involved in the suppression of angiogenesis in the tumor vasculature induced by the survivin/CCL21 DNA vaccine was shown by the finding that cultured murine endothelial cells expressing survivin were specifically lysed *in vitro* only by CD8$^+$ T cells isolated from mice successfully immunized with the survivin- or CCL21-based DNA vaccine (Fig. 4*C*). Typically, this lysis

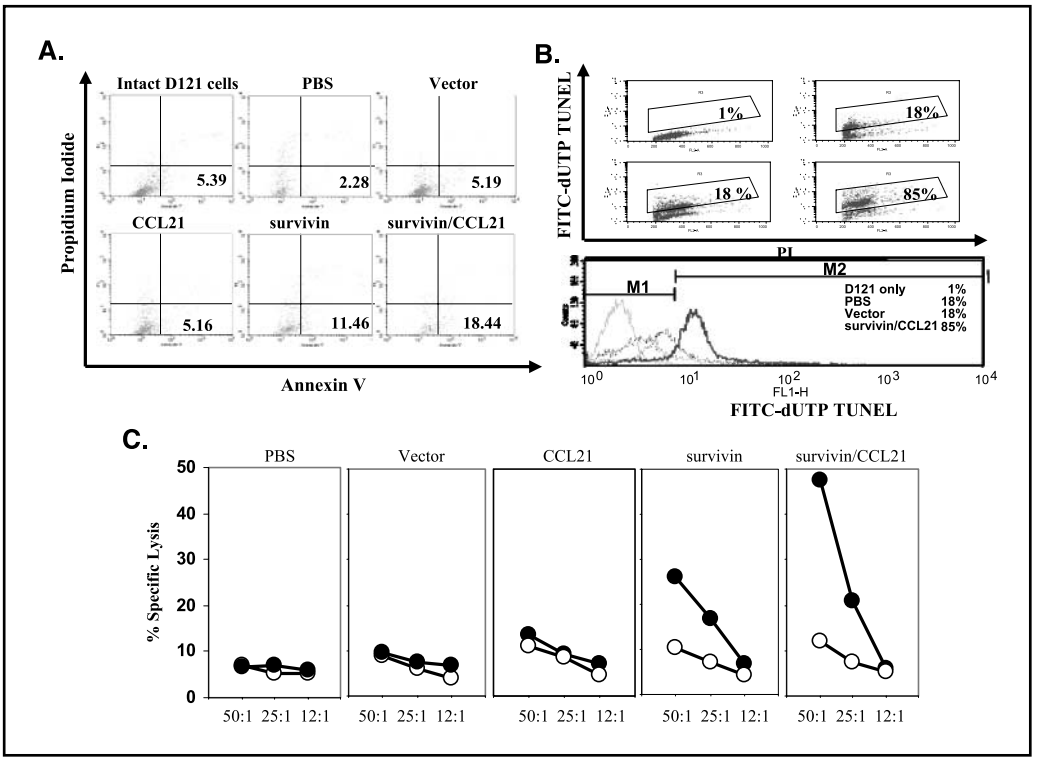

Figure 3. Analyses of apoptosis and *in vitro* cytotoxicity after immunization with survivin/CCL21-based DNA vaccines and various controls. All animals were challenged with D121 tumor cells after three immunizations with each individual DNA vaccine. Splenocytes were harvested 1 week after tumor cell challenge and incubated with 5×10^5 D121 tumor cells. *A,* adherent tumor cells were separated from splenocytes in suspension after a 3-hour coincubation and subjected to FACS analyses. ANNEXIN V-FITC was used to determine the percentage of cells within the population that are actively undergoing apoptosis at an early stage (3 hours). Propidium Iodide (*PI*) was used to distinguish viable from nonviable cells. *B,* late-stage tumor cell apoptosis (24 hours) induced by the survivin/CCL21 vaccine measured by the FITC-dUTP TUNEL assay. *C,* T cell–mediated cytotoxicity induced by various DNA vaccines against D121 lung cancer cells (●). Splenocytes were isolated 4 days after vaccination and analyzed for their lytic activity in a 4-hour ^{51}Cr release assay and D121 cells were used as targets for splenocytes obtained from mice treated with either PBS, empty vector, CCL21, survivin, or survivin/CCL21, respectively. Inhibition experiments were performed in the presence of 10 μg/mL mAbs directed against $H2K^b/H\text{-}2D^b$ MHC class I antigens (○). *Points,* mean for three mice.

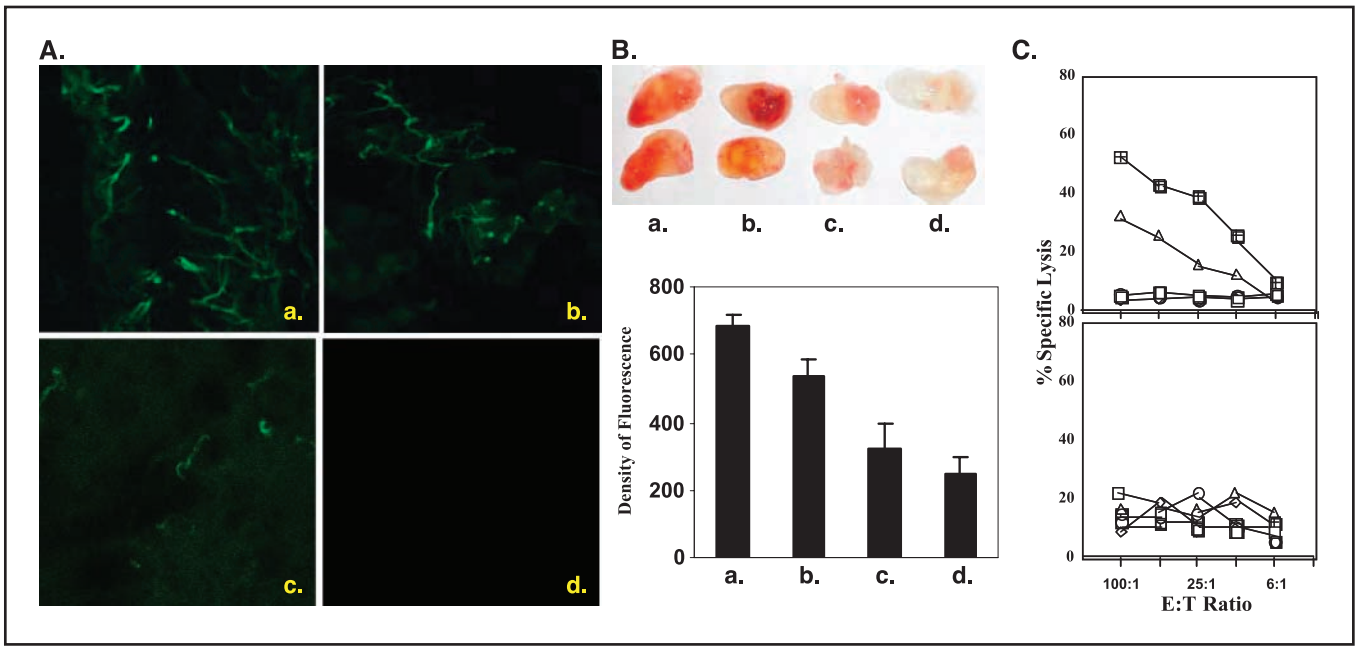

Figure 4. Suppression of angiogenesis by the survivin/CCL21 vaccine. *A,* angiogenesis was determined by the Matrigel assay. Quantification of vessel growth and staining of endothelium were achieved by fluorimetry and confocal microscopy, respectively using FITC-labeled Isolectin B4. Confocal images depicted (*a-d*) are PBS, empty vector control, survivin, and survivin/CCL21. *B,* average fluorescence of 100 μg Matrigel plugs from each experimental group of mice is depicted by the bar graphs ($n = 4$; mean ± SD; $P < 0.01$) where *a-d* are the same as listed in *A*. *C,* CTL- mediated killing of murine endothelial cells. The murine endothelial cell line HEV expressing survivin (*top*) was used as a target for splenocytes obtained from mice treated with either of the following: empty vector (□), CCL21 (○), survivin (△) and survivin/CCL21 (⊠), respectively. Inhibition experiments (*bottom*) with Abs against $H\text{-}2K^b/H\text{-}2D^b$ MHC class I antigens similar, as described in Fig. 3*C*.

was also found to be MHC class 1 antigen-restricted since antibodies against H-2K^b/H-2D^b antigens completely ablated all cytotoxic activity (Fig. 4*C*).

Vaccination Does not Impair Wound Healing or Fertility. We did not observe any statistically significant prolongation in the time required to completely heal small wounds inflicted on the backs of mice immunized with DNA vaccines encoding either survivin/CCL21, survivin, or CCL21 versus that of mice given only PBS (Fig. 5*A*). There also was no detectable difference in macroscopically visible swelling and inflammation in the wound areas of the three experimental groups. Additional experiments did not show any impact of the survivin- to CCL21-based DNA vaccine on fertility of the treated animal (Fig. 5*B* and *C*). This was based on the time elapsed from the start of cohabitation until parturition and on the number of pups born.

The Survivin Vaccine Activates Immune Effector Cells. We conclude that the antitumor response evoked by the survivin/CCL21 DNA vaccine, particularly the induction of an effective CTL response shown by the data depicted in Figs. 3*C* and 4*C*, suggests that the immune effector cells involved are activated. This contention was further supported by analyses of the activation markers of effector T cells at different levels of protein expression by double or triple staining during flow cytometric analyses. Three lines of evidence suggested that multiple effector cells and effector mechanisms are involved in this vaccine-induced activation. First, only the survivin/CCL21 vaccine per se was most effective in up-regulating the expression of CD25, CD28, and CD69 T cell activation markers (Fig. 6*A*). Second, the up-regulation of CD28 is of particular importance since its interactions with B7 costimulatory molecules on DCs is essential to achieve critical and multiple interactions between naïve T cells and antigen-presenting DCs. In contrast, the DNA vaccines encoding only survivin or CCL21 per se increased the expression of the T cell activation markers to a far lesser extent (Fig. 6*A*). Third, activation of both CD4$^+$ and CD8$^+$ T cells by the survivin/CCL21 vaccine was also indicated by their increase in intracellular proinflammatory cytokines IFN-γ and interleukin 2. In comparison, PBS and empty vector controls as well as DNA vaccines encoding solely survivin or CCL21 were found to be less effective in inducing these cytokines (Fig. 6*B*).

The up-regulated expression of ICAM-1, CD80, and DEC205 on DCs, achieved by the survivin/CCL21-based DNA vaccine could be particularly important because it is well known that the activation of T cells critically depends on effective cell-cell interactions with these costimulatory molecules expressed on DCs in order to achieve optimal ligation with T cell receptors. Again, immunization with doubly attenuated *Salmonella typhimurium* carrying eukaryotic plasmids encoding survivin/CCL21 induced the most pronounced up-regulation of these activation markers which was up to 2- to 3-fold higher than those of controls (Fig. 7).

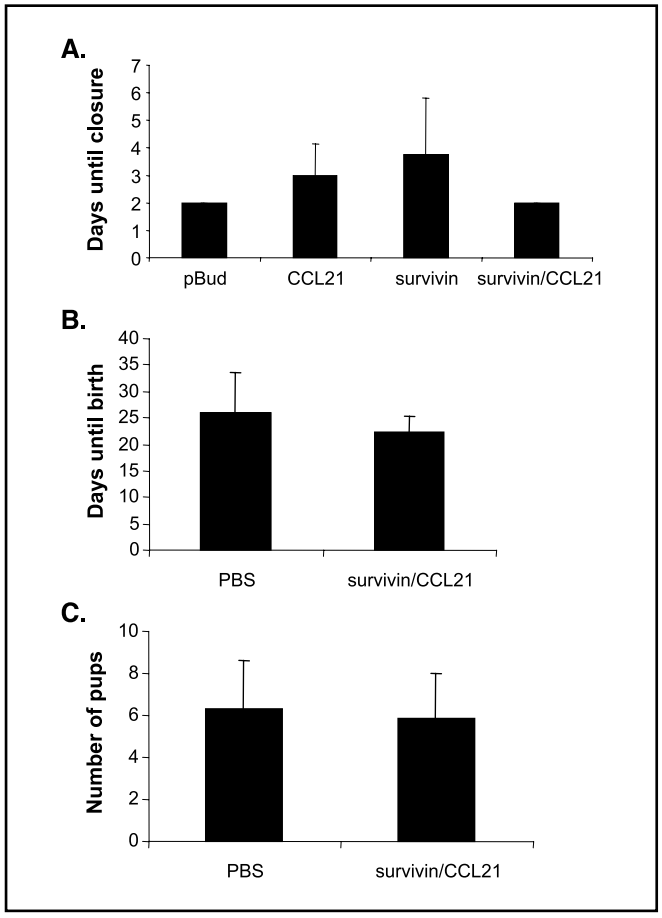

Figure 5. Effect of vaccination on wound healing and fertility. *A*, one wound of 3-mm length was inflicted on the upper backs of C57BL/6J mice (n = 4), 1 week after the last of three immunizations of each experimental group and the time of wound closure observed. Fertility was evaluated 1 week after the 3rd immunization with the survivin/CCL21 vaccine or PBS. Female C57BL/6J mice (n = 6) were allowed to cohabitate with males at a 3:1 breeding ratio and days until parturition (*B*) and number of pups (*C*) were noted.

Discussion

We showed for the first time that an oral DNA vaccine against survivin, coexpressing chemokine CCL21, could induce a CTL response sufficiently effective to attack tumor cells as well as suppress angiogenesis in the tumor neovasculature and result in the eradication or suppression of pulmonary lung tumor metastases in prophylactic and therapeutic settings.

It is well established that the disregulation of apoptosis by inhibitors of apoptosis protein, resulting in increased resistance to programmed cell death, is a common feature of malignant cells and represents a significant obstacle for successful prevention and therapy of cancer (14, 16, 21, 22, 24). It is also well known, particularly through the pioneering efforts of Folkman et al., that the suppression of angiogenesis in the tumor neovasculature provides a strong stimulus for eradication of tumor growth and that this offers a powerful tool for improving both cancer prevention and therapy (24, 27, 28). The inhibitor of apoptosis protein survivin was strongly implicated as an effective target to overcome such obstacles to cancer prevention and treatment (16, 21, 24). Indeed, several approaches, other than DNA-based vaccines, have been reported to target survivin. These include the application of dominant-negative mutants to initiate apoptosis via the mitochondrial pathway and suppression of tumor-associated angiogenesis (24, 39), blockage of survivin expression by antisense constructs (40, 41), induction of a CTL response against peptides of the survivin molecule (42, 43), and interference with survivin signaling pathways by molecular antagonists (24, 44).

However, despite these intensive efforts and the considerable interest in survivin as a direct target for cancer therapy, some critical aspects involved in the genetic immunization against

survivin remain to be explored. In fact, we could show here that an orally delivered DNA vaccine encoding survivin and secretory chemokine CCL21 can indeed induce a T cell–mediated antitumor immune response against established pulmonary metastases of non–small cell lung carcinoma, sufficiently effective to cause their ablation in mouse tumor model systems through mechanisms involving both suppression of tumor angiogenesis and induction of tumor cell apoptosis. We conclude that the immunologic mechanism(s) involved in the suppression of tumor cell angiogenesis involves $CD8^+$ T cell–mediated lysis that was likely facilitated by the overexpression of survivin by proliferating endothelial cells in the tumor vasculature triggered by vascular endothelial growth factor produced by tumor cells (25, 26). Two lines of evidence further support the contention that the suppression of tumor cell angiogenesis induced by our vaccine occurred via specific CTL-mediated killing of proliferating endothelial cells in the tumor vasculature. First, a distinct suppression of vascular endothelial growth factor–induced angiogenesis was shown *in vivo* by Matrigel assays and, second, specific CTL-mediated killing of both tumor cells and mouse endothelial cells overexpressing survivin was found repeatedly to be effective *in vitro* in cytotoxicity assays.

The contention that activation of the tumor cell death machinery occurred via CTL-mediated tumor cell lysis induced by our vaccine is supported by our data indicating robust tumor cell apoptosis by Annexin V and TUNEL assays and by several recent reports in the literature. First, CTLs were found to induce apoptosis of tumor cells by releasing cytolytic granules containing the pore-forming protein perforin as well as granzyme serine proteases, both known to be involved in CTL-mediated tumor cell lysis (45). Second, several groups of investigators reported that T cells mount a vigorous cytolytic response against survivin peptides *in vitro* and *in vivo* (43, 46), and that HLA class 1 antigen–restricted T cells against survivin exist in patients with various malignancies (43). Recently, survivin also was found to be immunogenic in colorectal cancer patients and to elicit $CD8^+$ and $CD4^+$ T cell–mediated responses (47). Third, prior observations suggested that overexpression of survivin in proliferating endothelial cells in different vascular beds can result in cytoprotective mechanisms counteracting apoptosis by reducing the generation of active caspases and hence preservation of cell survival (24, 26). However, we cannot rule out the contribution of the apoptotic mechanism involving a mitochondria-dependent pathway with cytochrome *c* being released from these organelles

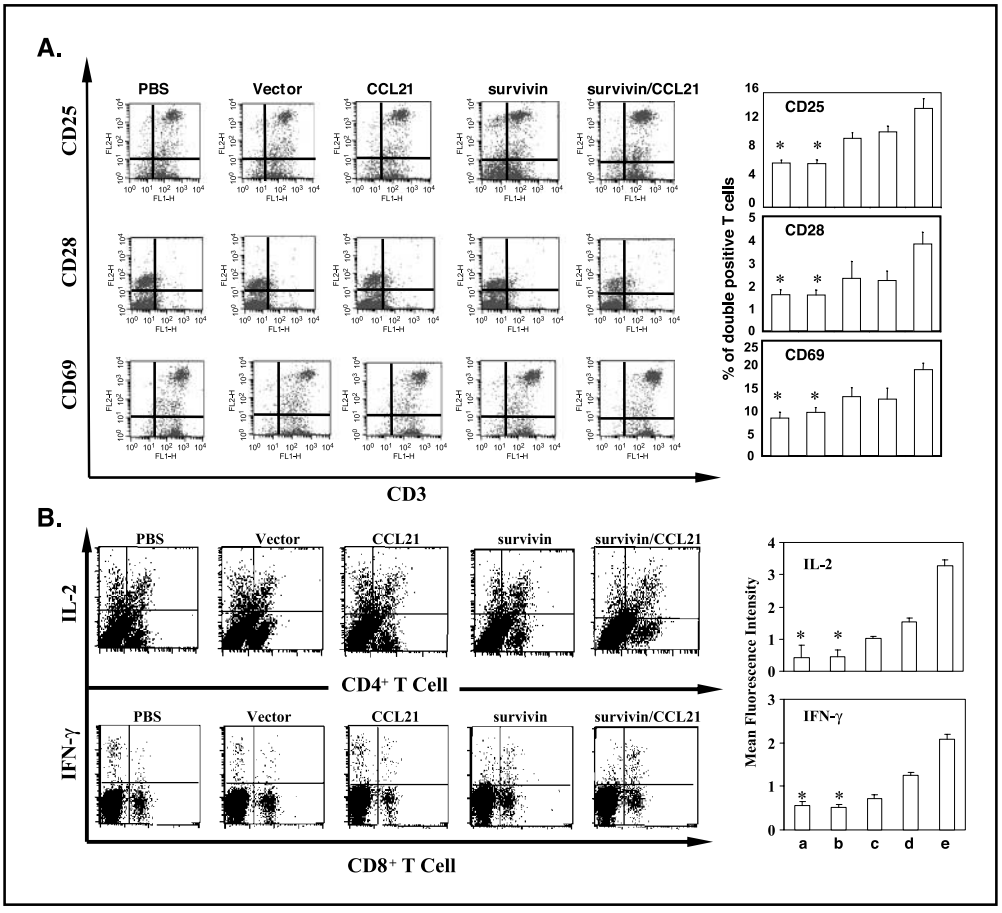

Figure 6. Analyses of CTL activation markers. *A,* upregulated expression of T cell activation molecules following three immunizations of C57BL/6J mice with the various DNA vaccines and the control vaccine indicated, followed by challenge with 1×10^5 D121 tumor cells. FACS analyses are depicted with splenocytes from such immunized mice 1 week after tumor cell challenge. Two-color flow cytometry analyses are shown with single cell suspensions of splenocytes. Anti-CD25, CD28, and CD69 mAbs were used in PE-conjugated form in combination with FITC-conjugated anti-mouse CD3 mAb. *B,* induction of intracellular cytokine release. Splenocytes were obtained 1 week after tumor cell challenge, stained with FITC-conjugated anti-CD4 or anti-CD8 mAbs, and fixed, permeabilized, and subsequently stained with PE-conjugated anti-IFNγ or anti-IL2 Abs. Cells stained with two colors were analyzed by flow cytometric analysis. *Columns,* mean for four animals; *bars,* SD. (*a*-*e*) PBS, empty vector, CCL21, survivin, and survivin/CCL21 vaccines. *, $P < 0.05$ compared with treatment groups.

into the cytosol, where it binds the protease activator, Apaf1, thus leading to the activation of caspase-9 (18, 24, 48).

In summary, our findings indicate that tumor cell death triggered by an oral, survivin-based DNA vaccine in both prophylactic and therapeutic settings, can be attributed to at least three mechanisms. First, proof of concept was established that the vaccine induced an effective $CD8^+$ T cell–mediated tumor-protective immune response. Second, it was shown that oral delivery of both the DNA vaccine and its essential adjuvant, chemokine CCL21, to secondary lymphoid organs by attenuated *S. typhimurium* is required to attain the activation of both T and dendritic cells necessary to achieve an effective tumor-protective immunity. Third, and most important, our DNA vaccine could induce a CTL response sufficiently effective to jointly trigger suppression of angiogenesis in the tumor vasculature and induction of tumor cell apoptosis which then combined to eradicate or suppress established pulmonary metastases of non–small cell lung carcinoma. It is anticipated that novel strategies such as this will serve as a basis for the rational design of future strategies, which will ultimately lead to further improvements in the prevention and treatment of cancer.

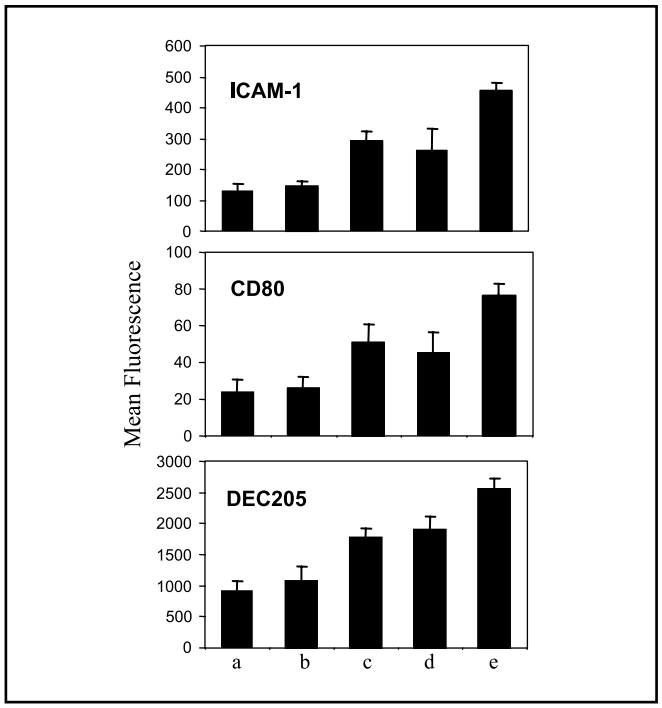

Figure 7. DNA vaccination enhances expression of costimulatory molecules by DCs. Similar as outlined in Fig. 6, multicolor flow cytometric analyses were performed with single cell suspensions of splenocytes obtained 1 week after tumor cell challenge. Splenocytes are stained with FITC labeled anti-CD11c Ab, in combination with either PE conjugated anti-ICAM-1, CD80, or DEC205 Abs together with biotinylated anti-IA^b Ab, followed by streptavidin-allophycocyanin. DC surface expressions of costimulatory molecules ICAM-1, CD80, and DEC205. *a-c,* PBS, empty vector, CCL21, survivin, and survivin/CCL21 vaccines, respectively. *Columns,* mean for four animals; *bars,* SD.

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T cell–mediated suppression of angiogenesis results in tumor protective immunity

He Zhou, Yunping Luo, Masato Mizutani, Noriko Mizutani, Ralph A. Reisfeld, and Rong Xiang

Antiangiogenic intervention is known to inhibit tumor growth and dissemination by attacking the tumor's vascular supply. Here, we report that this was achieved for the first time using an oral DNA minigene vaccine against murine vascular endothelial growth factor receptor 2 (FLK-1), a self-antigen overexpressed on proliferating endothelial cells in the tumor vasculature. Moreover, we identified the first H-2D^b–restricted epitope, FLK$_{400}$ (VILTNPISM), specifically recognized by cytotoxic T lymphocytes (CTLs). Such CTLs were capable of killing FLK-1$^+$ endothelial cells, resulting in suppression of angiogenesis and long-lived tumor protection. The specificity of this immune response was indicated because the DNA vaccine encoding the entire FLK-1 gene also induced a FLK$_{400}$-specific CTL response. This minigene vaccine strategy provides a more flexible alternative to whole-gene vaccination and facilitates in-depth mechanism studies to tailor DNA vaccines for optimal T-cell activation and tumor protection. (Blood. 2005;106: 2026-2032)

Introduction

Antiangiogenic intervention, which inhibits tumor growth by attacking the tumor's vascular supply, was pioneered by Folkman and colleagues,[1-3] who established that angiogenesis has a central role in the invasion, growth, and metastasis of solid tumors.[2,4] In fact, angiogenesis is a rate-limiting step in the development of tumors because tumor growth is generally limited to 1 to 2 mm^3 in the absence of a blood supply,[5] and beyond this minimum size, tumors often become necrotic and apoptotic.[6]

Vascular endothelial growth factor (VEGF) and its receptor tyrosine kinases play vital roles in angiogenesis.[7,8] Expression of murine VEGF receptor 2 (VEGFR2, also known as FLK-1), which binds the 5 isomers of murine VEGF, is restricted to endothelial cells and is up-regulated once these cells proliferate during angiogenesis in the tumor vasculature.[4,7,8] In fact, several approaches have been used to block FLK-1, including dominant-negative receptor mutants, germline disruption of VEGFR genes, monoclonal antibodies against VEGF, and a series of synthetic receptor tyrosine kinase inhibitors.[9,10]

We first reported on an alternative strategy, namely, an oral DNA vaccine encoding the entire FLK-1 gene, which prevented effective angiogenesis and inhibited tumor growth largely by CD8$^+$ T cell-mediated immune responses. CD8$^+$ cytotoxic T lymphocytes (CTLs) have the ability to specifically detect and kill antigen-bearing cells. They recognize antigens in the form of 8 to 10 amino acid long peptides, presented to T-cell receptors (TCRs) on the cell surface as complexes with major histocompatibility complex (MHC) class I molecules. These peptides, usually referred to as CTL epitopes, are generated in the cytosol of cells after proteolytic processing of antigen by the proteasome.[11] One of the primary aims of tumor vaccines is to induce CD8$^+$ CTL responses against such epitopes to eradicate tumors and prevent their relapse. The induction of a more effective antigen-specific immune response by DNA vaccines requires optimization of the vaccine design, including novel approaches for vaccine delivery and effective antigen processing. Such strategies include the use of an oral carrier system with a double-attenuated strain of *Salmonella typhimurium* (*dam$^-$; AroA$^-$*), which delivers the DNA to secondary lymphoid organs for subsequent transcription, translation, and antigen processing.[12,13]

The application of minigene vaccines provides an attractive approach because of their ease of synthesis and manipulation. Moreover, in contrast to vaccines encoding entire genes, minigene vaccines can induce immune responses directed against specific antigen epitopes while avoiding the interference of nonrelevant antigen epitopes. Consequently, such vaccines lend themselves to in-depth studies of immunologic mechanisms far more readily than DNA vaccines encoding entire genes. In this regard, several minigene strategies were reported to induce effective antitumor responses by an HLA-A2–restricted melan-A peptide analog epitope,[14] an HLA-A2–restricted carcinoembryonic antigen epitope,[15] and H-2D^b/K^b-restricted melanoma antigen epitopes.[13] Here, we identify the first H-2D^b–restricted FLK-1 epitope and demonstrate that a novel minigene DNA vaccine protects mice from tumors of different origins by inducing a T cell-mediated suppression of tumor angiogenesis.

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H.Z. designed and performed the research, analyzed data, and wrote this manuscript. Y.L., M.M., and N.M. contributed considerably to the design and performance of the study. The contributions of R.A.R. and R.X. included experimental designs and manuscript preparation.

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Materials and methods

Animals, bacterial strains, and cell lines

Male or female C57BL/6J mice were purchased from the Jackson Laboratory (Bar Harbor, ME). All animal experiments were performed according to the National Institutes of Health Guides for the Care and Use of Laboratory Animals, and all protocols were approved by the Institutional Animal Care and Use Committee (IACUC) of The Scripps Research Institute.

The murine lung carcinoma cell line D121 was provided by Dr L. Eisenbach (Weizmann Institute of Science, Rehovot, Israel). The murine prostate cancer cell line RM9 was obtained from Dr T. C. Thompson (Baylor College of Medicine, Houston, TX). The murine breast cancer cell line EO771 was kindly made available by Dr D. Ross (University of Kentucky, Louisville, KY). Murine endothelial cell line, MS1, was purchased from the American Type Culture Collection (ATCC; Rockville, MD). All cell lines were cultured in Dulbecco modified Eagle medium (Invitrogen, Grand Island, NY), supplemented with 10% (vol/vol) fetal bovine serum.

The double-attenuated *S typhimurium* (*AroA*$^-$, *dam*$^-$) strain RE88 was kindly provided by the Remedyne Corporation (Santa Barbara, CA) and was transformed with DNA vaccine plasmids as previously described.[16]

Construction of expression vectors

The expression vector pCMV/ER/Myc was purchased from Invitrogen (Carlsbad, CA). Vector construction is illustrated schematically in Figure 1A. The following expression vectors were constructed: pHI-myc, pHI-Db-myc, pHI-Kb-myc, where the HIVtat peptide (HI) represents RKKRRQRRR. The FLK_{94}, FLK_{400}, and FLK_{1210} peptides stand for RVVGNDTGA, VILTNPISM, and FHYDNTAGI, respectively. FLK_{54}, FLK_{771}, and FLK_{1129} peptides are designated for RGQRDLDWL, VIAMFFWLL, and TTPEMHYQTM, respectively. All peptides were engineered to be in-frame with the myc epitope. Constructs were confirmed by DNA sequencing at the Scripps Research Institute's Core Facility (La Jolla, CA). Peptide expression was demonstrated by Western blotting with monoclonal anti-myc antibody (Invitrogen, Carlsbad, CA). Once peptide expression was verified, a stop codon was introduced immediately in front of the *myc* epitope sequences. The resulting vectors, namely pHI, pHI-Db, and pHI-Kb, were verified by nucleotide sequencing and used to transform double-attenuated *S typhimurium* (*dam*$^-$, *AroA*$^-$) for immunization. The pCMV empty vector was also included in the experiments as a control.

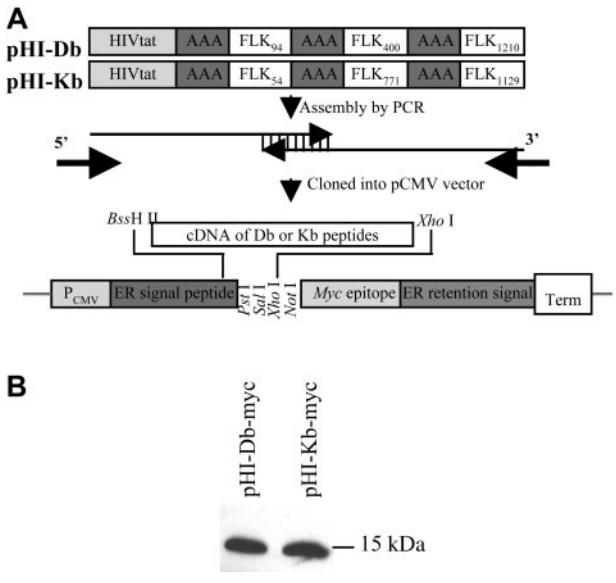

Figure 1. Construction of the FLK-1 DNA minigene vaccine. (A) Minigenes encoding the HIVtat translocation peptide, a spacer (AAA), and murine FLK-1 H-2D^b– and K^b-restricted epitopes (pHI-Db or pHI-Kb, respectively), were assembled by polymerase chain reaction (PCR) with overlapping oligonucleotides as templates. D^b-restricted epitopes include FLK_{94}: RVVGNDTGA; FLK_{400}: VILTNPISM; FLK_{1210}: FHYDNTAGI. K^b-restricted epitopes include FLK_{54}: RGQRDLDWL; FLK_{771}: VIAMFFWLL; FLK_{1129}: TTPEMYQTM. The PCR fragments generated were cloned into a pCMV vector at C-terminal of ER signal peptide (endoplasmic reticulum) by using *Bss*H II and *Xho*I restriction sites. (B) Proteins encoded by minigenes were expressed in mammalian cells. This was indicated when 293T cells were transfected with either pHI-Db-myc or pHI-Kb-myc for 24 hours, harvested, lysed, and analyzed by Western blotting with anti-myc monoclonal antibody.

Peptide synthesis

All peptides were synthesized with more than 95% purity by high-performance liquid chromatography (HPLC) by Multiple Peptide Systems (San Diego, CA).

Oral immunization and tumor-cell challenge

Groups of C57BL/6J mice were immunized 3 times at 1-week intervals by gavage with 100 μL phosphate-buffered saline (PBS) containing approximately 5×10^8 double-attenuated *S typhimurium* harboring either pCMV, pHI, pHI-Db, or pHI-Kb plasmids. Mice were challenged intravenously with different carcinoma cells 2 weeks after the last immunization.

Cytotoxicity and ELISPOT assays and in vivo depletion

Cytotoxicity was measured by a standard ^{51}Cr-release assay as previously described.[15] The percentage of specific target cell lysis was calculated by the formula $[(E\text{-}S)/(T\text{-}S)] \times 100$, where E is the average experimental release, S the average spontaneous release, and T the average total release.

Enzyme-linked immunospot (ELISPOT) assays were performed with an ELISPOT kit (PharMingen, La Jolla, CA) according to the instructions provided by the manufacturer.

In vivo depletion was performed on vaccinated mice by intraperitoneal injection of anti-CD4 antibody (GK1.5, 0.4 mg/mouse) or anti-CD8 antibody (2.43, at 0.6 mg/mouse) 1 day before tumor challenge and repeated weekly.

Evaluation of antiangiogenic effects

Two weeks after the last vaccination, mice were given subcutaneous injections in the sternal region with 400 μL growth factor-reduced Matrigel (BD Biosciences, San Jose, CA) containing 400 ng/mL basic fibroblast growth factor (PeproTech, Rocky Hill, NJ). In all mice, the endothelium was stained 6 days later by intravenous injection of 200 μL fluorescent *Bandeiraea simplicifolia* lectin I, isolectin B4 at 0.1 mg/mL (Vector Laboratories, Burlingame, CA). Fifteen minutes later, Matrigel plugs were excised and evaluated by confocal microscopy (Axiovert 100TV microscope; Carl Zeiss, Oberkochen, Germany; 40×/1.3 NA objective; and SPOT camera and software), and then lectin-fluorescein isothiocyanate (FITC) was extracted with RIPA lysis buffer (0.15 mM NaCl/0.05 mM Tris [tris(hydroxymethyl)aminomethane]–HCl, pH 7.2/1% Triton X-100/1% sodium deoxycholate/0.1% sodium dodecyl sulfate) from 100-μg Matrigel plugs to be quantified by fluorometry at 490 nm.

Statistical analysis

The statistical significance of differential findings between experimental groups and controls was determined by the Student *t* test. Findings were regarded as significant when 2-tailed *P* was less than .05.

Results

Minigenes encoded by expression vectors are expressed in mammalian cells

We previously demonstrated that a DNA vaccine encoding the entire murine FLK-1 gene effectively induced CD8$^+$ T cell-mediated antiangiogenesis that protected mice from tumor-cell

challenge.[17] Here, a minigene approach was adopted to identify the specific CTL epitopes involved to conduct in-depth mechanistic studies and to test our hypothesis that vaccination with such epitopes can induce similar antiangiogenic responses as the whole-gene vaccine. To this end, 3 peptides were included in H-2D^b– or H-2K^b–restricted minigenes based on the binding predicted for these MHC class I molecules by the HLA Peptide Binding Predictions program provided by the BioInformatics & Molecular Analysis Section (BIMAS) of the National Institutes of Health (NIH), website: http://bimas.dcrt.nih.gov/molbio/hla_bind/.

Expression vectors were constructed based on the backbone of pCMV/ER/Myc (Figure 1A). A HIVtat peptide (RKKRRQRRR), one of the commonly used membrane-translocating peptides,[18-20] is also included in our minigene vaccine to facilitate the delivery of the encoded peptides as previously demonstrated.[15,20] After transfection of 293T cells with either pHI-myc, pHI-Db-myc, or pHI-Kb-myc, correct expression of these constructs was demonstrated by Western blotting, which revealed single bands with the expected molecular mass of 15 kDka (Figure 1B). The mature peptides did not contain the myc epitope because the vaccine vectors pHI, pHI-Db, and pHI-Kb were generated by introducing a stop codon immediately downstream from the peptide-coding sequences. The correct vector constructs were confirmed by DNA sequencing. The empty pCMV vector was also included for control purposes.

The pHI-Db minigene vaccine protects mice against tumors of different origin by inducing immune responses that suppress tumor angiogenesis

Initially, we tested the minigene DNA vaccines in a prophylactic lung cancer model, where mice were first vaccinated with the minigene vaccines and then challenged intravenously with D121 lung carcinoma cells. In this case, the pHI-Db minigene elicited the best tumor protection with 62.5% of mice surviving 75 days after tumor cell challenge (Figure 2A). In contrast, none of the mice in the pCMV control group survived and the pHI or pHI-Kb vaccines induced only minimal tumor protection with 25% of the mice surviving 75 days after tumor challenge (Figure 2A).

To verify that the minigene vaccine effectively protects mice from tumors of different origins because it was designed for antiangiogenesis purposes, the vaccine efficacy was also tested in a RM9 prostate carcinoma model. In this case, the pHI-Db minigene also protected the mice from RM9 tumor cell challenge (Figure 2B), suggesting that the pHI-Db vaccine induces suppression of metastases independent of the tumor type.

In vivo depletion assays were performed to identify the cell population responsible for the tumor protection effects. Depletion of CD8 cells completely abrogated the vaccine-induced protection, whereas the depletion of CD4 cells moderately enhanced the protection against tumor challenge (Figure 2C), suggesting the CD8 T cells are the major effectors.

The specificity of the CTL responses was further investigated in ^{51}Cr-release assays. The pHI-Db vaccine induced a specific cytotoxic response against a FLK-1$^+$ (Figure 3A) endothelial cell line MS1 (Figure 3B), but not against FLK-1$^-$ (Figure 3A) RM9 prostate carcinoma cells (Figure 3C). These data suggest that the cytotoxic response induced by the pHI-Db vaccine was indeed directed against endothelial cells, presumably specific for FLK-1, rather than against tumor cells. This finding suggests that the pHI-Db minigene vaccine would induce an antiangiogenic response in vivo.

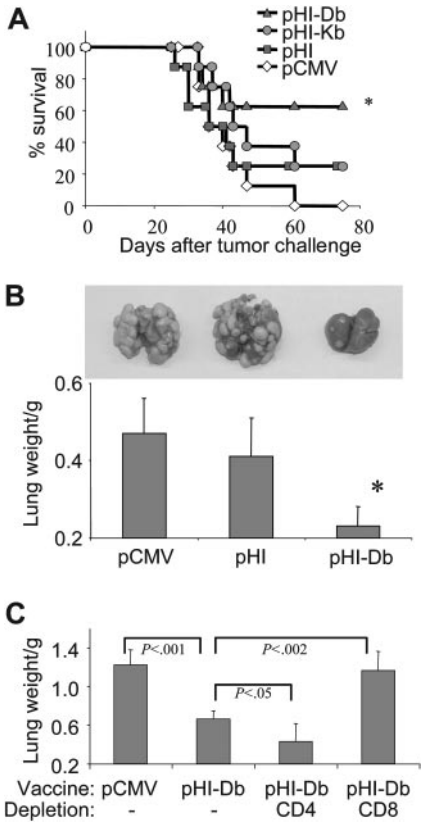

Figure 2. DNA minigene vaccine pHI-Db protects mice from tumor challenges. (A) Groups of C57BL/6 mice (n = 8) were immunized 3 times at 1-week intervals with attenuated *S typhimurium* harboring the vectors indicated. Empty diamonds indicate the pCMV control group; shaded squares show the pHI control group; red triangles depict the pHI-Db group; and green circles stand for the pHI-Kb groups. Mice were challenged intravenously 2 weeks after the last immunization with 1×10^5 D121 lung carcinoma cells and monitored for survival until 75 days after tumor challenge. *$P < .02$ compared to pCMV control group. (B) Vaccinated mice were challenged intravenously 2 weeks after the last immunization with 1×10^5 RM9 prostate carcinoma cells. Mice were killed 28 days after tumor cell challenge and lung weights assessed. The top panel depicts representative lungs and the bottom panel shows average lung weights. Normal lung weight is about 0.2 g. *$P < .001$ and .001 compared to pCMV and pHI, respectively. Experiments were repeated twice with similar results. (C) In vivo depletion was performed as described in "Materials and methods." Mice were challenged intravenously with 2.5×10^5 tumor cells and killed 23 days thereafter. Error bars indicate standard deviation (SD).

We further proved this hypothesis by performing Matrigel assays, which indicated that vaccination with minigene pHI-Db indeed suppressed vascularization. This was clearly demonstrated by reduced blood vessel formation observed in representative Matrigel plugs after in vivo staining of endothelium with FITC-conjugated lectin (Figure 4A). This difference in vessel formation was also demonstrated quantitatively by measuring the average relative fluorescence (Figure 4B). Taken together, these findings demonstrate that the pHI-Db minigene vaccine induced antiangiogenic effects, which protected mice from challenge with tumor cells of different origin.

The pHI-Db vaccine induces a FLK$_{400}$-specific immune response

To evaluate each of the 3 peptides encoded by the pHI-Db minigene, splenocytes isolated from mice immunized with pHI-Db were analyzed by ELISPOT assays using individual synthetic

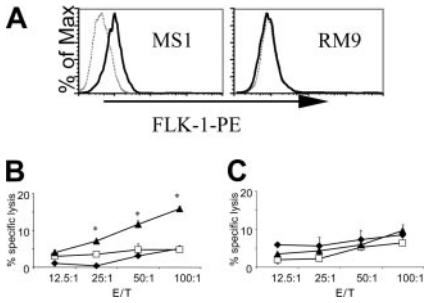

Figure 3. The DNA minigene vaccine pHI-Db induces specific CTL killing of FLK-1$^+$ endothelial cells but not of FLK-1$^-$ tumor cells. (A) Surface expression of FLK-1 by endothelial cell line MS1 and murine prostate carcinoma cell line RM9. Gray dotted lines indicate phycoerythrin (PE)–conjugated isotype control antibody; black solid lines, PE-conjugated anti–FLK-1. Groups of immunized C57BL/6J mice (n = 4) were killed 2 weeks after the last immunization and isolated splenocytes were stimulated with irradiated MS1 cells for 5 days. Thereafter, cytotoxicity assays were performed with MS1 (B) or RM9 (C) serving as target cells. ♦, pCMV control group; □, pHI group; ▲, pHI-Db group. Experiments were repeated 3 times with similar results. *$P < 0.001$ compared with pCMV or pHI control groups. E/T indicates the ratio of effector and target cells.

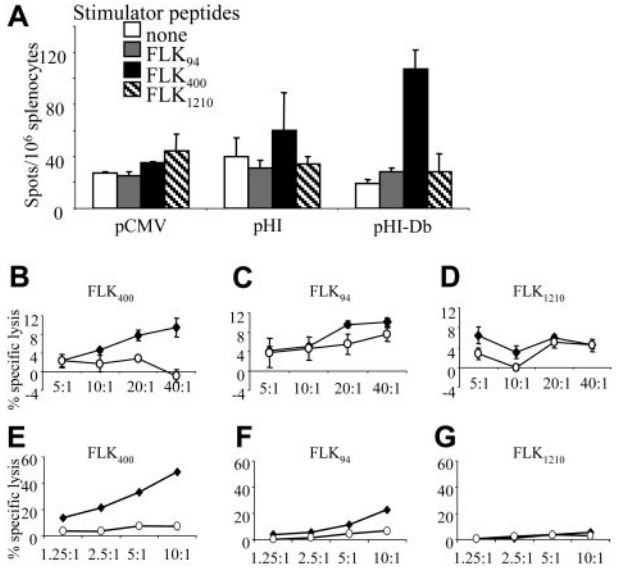

Figure 5. The pHI-Db minigene vaccine induces an H-2D^b–restricted, FLK$_{400}$–specific response. Groups of vaccinated C57BL/6 mice (n = 4) were killed 2 weeks after the last immunization. (A) ELISPOT assays were performed on splenocytes isolated by using either no stimulator or synthetic peptides FLK_{94} (25 μg/mL), FLK_{400} (10 μg/mL), or FLK_{1210} (25 μg/mL) as stimulators. (B-D) Isolated splenocytes were stimulated with FLK_{400} (B), FLK_{94} (C), or FLK_{1210} (D) peptides for 5 days. Thereafter cytotoxicity assays were performed with MS1 (♦) or RM9 (○) serving as target cells. Experiment was repeated twice with 2 μg/mL or 10 μg/mL stimulator peptides and with similar results. (E-G) Splenocytes isolated from pHI-Db–vaccinated mice were stimulated with FLK_{400} (E), FLK_{94} (F), or FLK_{1210} (G) peptides for 7 days, and restimulated twice weekly with irradiated FLK_{400}-loaded (E), FLK_{94}-loaded (F), or FLK_{1210}-loaded (G) splenocytes from normal C57BL/6 mice. Thereafter cytotoxicity assays were performed with MS1 (♦) or RM9 (○) serving as target cells. Error bars indicate SD.

peptides as stimulators. A specific FLK_{400} response was detected only in the pHI-Db–vaccinated group of mice (Figure 5A), whereas no significant FLK_{94}- or FLK_{1210}-specific responses were found in any of the experimental groups of mice, suggesting that FLK_{400} is the major epitope recognized by CTL effector cells.

To further strengthen this notion, splenocytes from vaccinated mice were stimulated with synthetic peptides for 5 days and tested against MS1 and RM9 target cells in cytotoxicity assays. Only FLK_{400}-stimulated splenocytes exhibited specific cytotoxic killing against FLK-1$^+$ MS1 target cells, but revealed almost no killing of FLK-1$^-$RM9 tumor cells (Figure 5B). Splenocytes stimulated with FLK_{94} induced low levels of MS1-specific killing (Figure 5C), whereas FLK_{1210}-stimulated splenocytes mainly displayed low levels of nonspecific killing (Figure 5D), confirming the dominance of the FLK_{400} epitope within the minigene vaccine.

When peptide-stimulated splenocytes isolated from pHI-Db–vaccinated mice were restimulated twice more in vitro with irradiated, peptide-loaded splenocytes every 7 days, and then tested again for their cytotoxicity, only those cells restimulated with FLK_{400}-loaded splenocytes showed greatly enhanced cytotoxicity, resulting in a higher percent specific killing at a much lower effector-target (E/T) ratio (Figure 5E). In contrast, such restimulation with FLK_{94}-loaded splenocytes resulted in a lower level of MS1-specific killing (Figure 5F), whereas cells restimulated with FLK_{1210}-loaded splenocytes failed to induce any significant killing (Figure 5G). Taken together, these findings suggest that restimulation with FLK_{400}-loaded splenocytes enriches the CTL population that specifically targets FLK-1$^+$ endothelial cells.

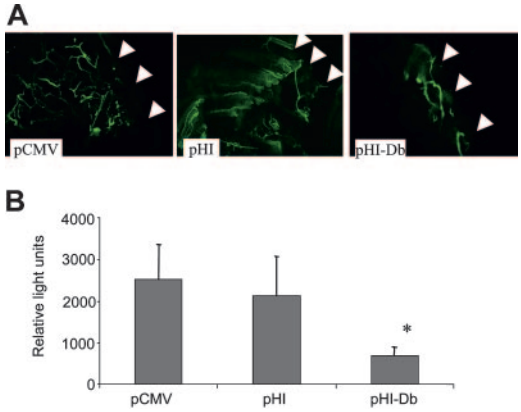

Figure 4. The pHI-Db minigene vaccine induced suppression of angiogenesis determined by Matrigel assay. Quantification of vessel growth and staining of endothelium was determined by fluorometry and confocal microscopy, respectively, using FITC-labeled isolectin B4. (A) Representative Matrigel plugs were examined by confocal microscopy (original magnification × 200; 1.3 NA). The arrows indicate the borders of the Matrigel plug. (B) The average fluorescence of Matrigel plugs from each group of mice is depicted by the bar graph (n = 4; mean + SD). *$P < .05$ pCMV or pHI groups. The experiment was repeated once with similar results.

To validate our hypothesis that the FLK_{400}-specific immune response contributed to the antitumor effects elicited by the pHI-Db vaccine, we assessed the tumor protective ability of a minigene vaccine encoding only FLK_{400} in the absence of FLK_{94} and FLK_{1210} and compared it with the effect of the pHI-Db minigene vaccine in an EO771 breast carcinoma model. EO771 cells do not express FLK-1, but express surface H-2D^b as detected by flow cytometry (Figure 6A). In fact, both pHI-Db and pHI-FLK_{400} minigene vaccines significantly protected the mice against EO771 tumor cell challenge and to an extent comparable to the protection induced by a DNA vaccine encoding the entire FLK-1 gene (Figure 6B). The pHI-Db vaccine also achieved similar efficacy as FLK-1 whole-gene vaccine in RM9 prostate and D121 lung carcinoma models (data not shown).

Long-term protection was established by the pHI-Db minigene vaccine; at 10 months after their last vaccination, pHI-Db–vaccinated mice showed significantly reduced lung metastases after intravenous challenging with EO771 breast carcinoma cells (Figure 6C), and FLK_{400}-specific T cells could still be detected in the spleen of these mice (Figure 6D).

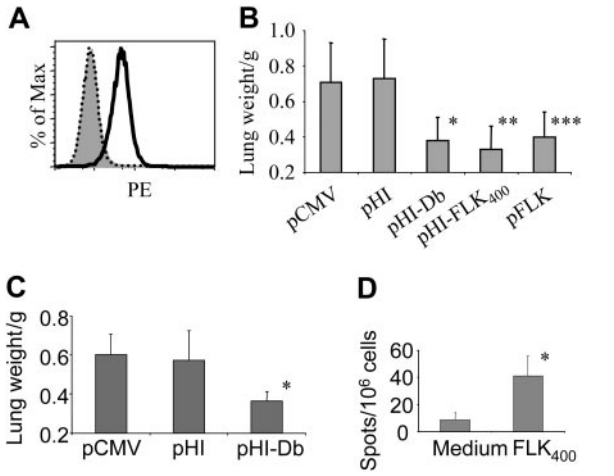

Figure 6. DNA minigene vaccine pHI-FLK$_{400}$ suppresses tumor metastasis and the immunity induced by pHI-Db is long-lasting. (A) Surface expression of FLK-1 (black dotted line) or H-2D^b (black solid line) of EO771 breast carcinoma cells. Isotype control is shown with gray shaded curve. (B) Groups of vaccinated mice (n = 8) were challenged intravenously with 2×10^5 EO771 breast carcinoma cells 2 weeks after the last vaccination. Mice were killed 21 days later and lung weights assessed. *$P < .01$ compared to pCMV and pHI; **$P < .005$ compared to pCMV and pHI; ***$P < .05$, $P < .05$, $P > .05$, and $P > .05$ compared to pCMV, pHI, pHI-Db, and pHI-FLK$_{400}$, respectively. (C) Groups of vaccinated mice (n = 4) were challenged intravenously with 2×10^5 EO771 breast carcinoma cells 10 months after the last vaccination. *$P < .01$ compared to the pCMV control group. (D) ELISPOT assay performed with splenocytes isolated from mice (n = 4) 10 months after pHI-Db vaccination in the presence or absence of FLK$_{400}$ peptide. *$P < .02$ compared to culture medium alone. Error bars indicate SD.

The DNA vaccine encoding the entire FLK-1 gene induces a FLK$_{400}$-specific CTL response

We confirmed that FLK$_{400}$ is indeed a true FLK-1 epitope by demonstrating that the FLK$_{400}$-specific response was induced by a DNA vaccine encoding the entire FLK-1 gene. Such responses were detected in splenocytes freshly isolated from pFLK-1–vaccinated mice as demonstrated by ELISPOT assays (Figure 7A). These splenocytes maintained the specificity of the responses after in vitro stimulation with FLK$_{400}$ peptides (Figure 7B). However, controls were negative because stimulation with FLK$_{94}$ had no effect when compared to nonstimulated cells, and only nonspecific activation resulted from stimulation with FLK$_{1210}$ (Figure 7B). Moreover, splenocytes isolated from pFLK-1–vaccinated mice also displayed preferential cytotoxic killing of EO771 tumor cells loaded with FLK$_{400}$ as compared to the killing of unloaded EO771 cells (Figure 7C). Similar results were also observed in pHI-Db–vaccinated mice (Figure 7D), which were used as a positive control. The killing of EO771 or FLK$_{400}$–loaded EO771 tumor cells was largely indistinguishable in pCMV or pHI control groups (Figure 7E-F). Taken together, these findings prove that the DNA vaccine encoding the entire FLK-1 gene was capable of inducing a FLK$_{400}$-specific immune response.

Discussion

There are several advantages in targeting CD8$^+$ T cells to proliferating endothelial cells in the tumor vasculature rather than directly to tumor cells. First, endothelial cells are genetically stable and do not down-regulate MHC class I antigen, an event that frequently occurs in solid human tumors and severely impairs T cell-mediated antitumor responses.[21] Second, immune suppression triggered by tumor cells in the tumor microenvironment can also be avoided by this approach. Third, the therapeutic target is tumor-independent, thus killing of proliferating endothelial cells in the tumor microenvironment can be effective against a variety of malignancies. Finally, proliferating endothelial cells are readily available to lymphocytes in the bloodstream and consequently CD8$^+$ T cells can reach the target tissues unimpaired by anatomic barriers such as the blood-brain barrier or encapsulation of tumor tissues.[22]

We took advantage of this approach as indicated by prior data from our laboratory, which demonstrated that an oral DNA vaccine encoding autologous FLK-1 prevents effective tumor angiogenesis and inhibits tumor growth and metastasis.[17] However, the full-length FLK-1 gene used in these studies was about 4 kb, encoding for a protein of approximately 190 kDa,[23] with its human counterpart being similar in size.[24] Because of the large size of such genes, mutations are likely to be introduced during vaccine production and in the host once plasmids encoding these genes are delivered by *S typhimurium* to secondary lymphoid tissues such as Peyer patches. Thus, safety and quality control issues will be of great

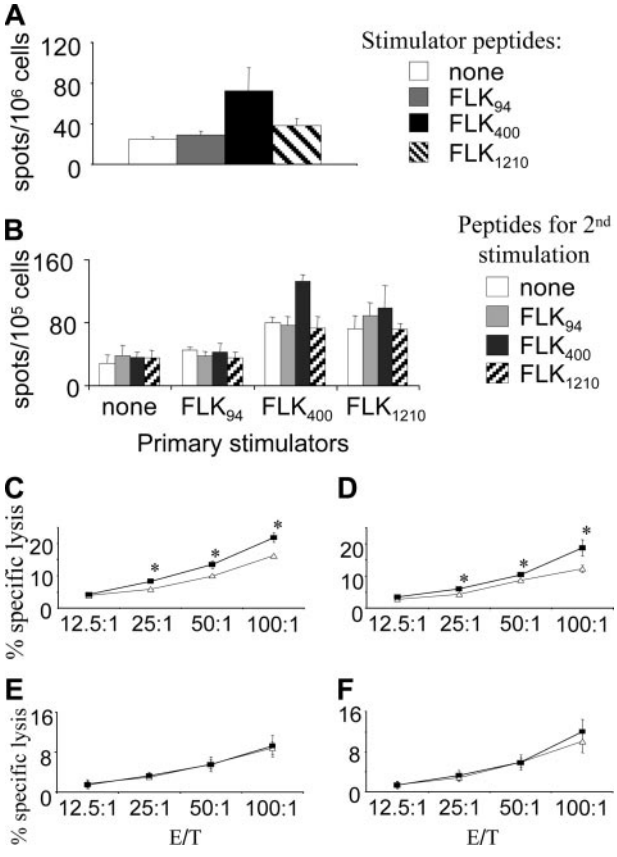

Figure 7. DNA vaccine encoding full-length FLK-1 induces FLK$_{400}$-specific responses. (A) ELISPOT assays performed with freshly isolated splenocytes from pFLK-1–vaccinated mice and stimulator with FLK$_{94}$, FLK$_{400}$, FLK$_{1210}$, or no peptide. (B) Splenocytes isolated from pFLK-1–vaccinated mice were first stimulated in vitro for 5 days with peptides indicated by "primary stimulators," then restimulated in ELISPOT assays. Stimulators used in such ELISPOT assays are unloaded, FLK$_{94}$-loaded, FLK$_{400}$-loaded, or FLK$_{1210}$-loaded splenocytes from normal C57BL/6 mice. Splenocytes from pFLK-1 (C), pHI-Db (D), pCMV (E), and pHI (F) groups of mice were stimulated with irradiated MS1 cells for 5 days, and cytotoxicity assays were performed against unloaded (△) or FLK$_{400}$-loaded (■) EO771 target cells. *$P < .02$ compared to unloaded EO771 target cells. The killing of FLK$_{94}$-loaded or FLK$_{1210}$-loaded EO771 cells was indistinguishable from that of unloaded EO771 cells (data not shown). This experiment was repeated once with similar results (data not shown). Error bars indicate SD.

concern before such approaches become clinically applicable. For this and other reasons, minigene vaccine approaches were used to create a simpler and more defined vaccine, which also facilitates the identification of specific FLK-1 epitopes recognized by CTLs for in-depth studies on vaccine mechanisms and efficacy. Here, we demonstrated for the first time that a FLK-1–based minigene, pHI-Db, induced $CD8^+$ T cell-mediated suppression of tumor angiogenesis and protected mice from carcinomas of different origins such as breast, prostate, and lung. Importantly, we identified FLK_{400} to be the major epitope recognized by CTLs, which mediated this tumor-protective effect. The pHI-Db vaccine-induced FLK_{400}-specific response was long-lasting because we still detected such specific T cells in the spleen 10 months after the vaccination. These FLK_{400}-specific T cells were very likely present in the periphery. In time of tumor challenge or relapse, on encounter of antigen, namely, FLK_{400} presented by a MHC class I molecule in the tumor microenvironment, these T cells were activated and proliferated and executed cytotoxic function. This can explain the long-term protection achieved by the minigene vaccine. Significantly, minigene vaccines pHI-Db and pHI-FLK_{400} showed an antitumor efficacy that was similar to that achieved by the DNA vaccine encoding the entire FLK-1 gene, thereby indicating that these minigene vaccines are promising alternatives.

It was previously reported that an orally delivered DNA minigene vaccine encoding murine melanoma peptide epitopes required poly-ubiquitination to lead to optimal antigen processing, which evoked a potent immune response.[13] Likewise, in our current experiments, antigen processing proved to be important because a minigene vaccine encoding the FLK_{400} peptide was most effective in protecting mice from tumor cell challenges when it also encoded the HIVtat peptide (data not shown). The rationale for using this HIVtat peptide in our minigene vaccine is based on the fact that it is one of the commonly used membrane-translocating peptides. Such translocating peptides are able to transport antigen peptides into the endoplasmic reticulum, in a transport-associated protein (TAP)–independent manner, where they then can be effectively processed and trimmed to become CTL epitopes.[18-20] Previously reported data from our laboratory also showed that the inclusion of this peptide in carcinoembryonic antigen-based DNA minigene vaccines induced effective CTL responses against the encoded CEA epitope.[15]

It is generally believed that $CD4^+$ T-cell help is required to overcome tolerance to effectively generate immune responses against weak self antigen-like tumor-associated antigen, and in most cases $CD4^+$ T cell help is required for the generation of long-lived, functional memory $CD8^+$ T cells.[25-28] In this regard, strategies such as fusion of tumor antigen to $CD4^+$ stimulators to provide cognate $CD4^+$ help in DNA vaccines have been reported.[29] The aim of our minigene vaccine strategy was to specifically activate antigen-specific $CD8^+$ T cells without providing a particular epitope for CD4 T cells. In fact, this strategy proved to be successful in inducing an effective CD8 immune response that efficiently induced long-term protection of mice from tumor cell challenges. It is possible that the administration of *S typhimurium* could induce activation of $CD4^+$ T cells that are specific for epitopes on these bacteria and such $CD4^+$ T cells could then provide the necessary help. In this regard, we found a slight up-regulation of activation markers on $CD4^+$ T cells in Peyer patches after administration of attenuated *S typhimurium* harboring empty vector as compared to the PBS control group (data not shown). It is also possible that the attenuated *S typhimurium* could elicit danger signals,[12] which directly activate antigen-presenting cells (APCs) and bypass the need for $CD4^+$ T cell-mediated licensing of APCs.[30] Moreover, it was recently suggested that a $CD4^+$ T-cell population, without activation, can provide an antigen nonspecific maintenance function for $CD8^+$ T-cell memory.[30] Consequently, the antigen-specific activation of $CD4^+$ T cells may not be crucial for the generation and maintenance of $CD8^+$ T-cell memory. The exact nature of $CD4^+$ T-cell help is difficult to demonstrate in our experimental system, because $CD4^+$ T cells also contribute to the negative control. Such an effect is presumably mediated by regulatory T cells, which inhibit immune responses to ensure self-tolerance.[31] In our experimental systems, the depletion of $CD4^+$ T cells in vaccinated mice resulted in improved protection against tumor challenge.

In summary, we reported here the first antiangiogenic minigene vaccine and identified the initial H-$2D^b$-restricted FLK-1 epitope-FLK_{400} (VILTNPISM). Importantly, the pHI-Db and pHI-FLK_{400} minigene vaccines achieved similar efficacy as the DNA vaccine encoding the entire FLK-1 gene, and thereby provided a safer, simpler, and more flexible alternative to the whole-gene vaccine, while adding a new dimension to antiangiogenic interventions in cancer immunotherapy.

Acknowledgments

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DNA-based vaccines activate innate and adaptive antitumor immunity by engaging the NKG2D receptor

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The interaction of NKG2D, a stimulatory receptor expressed on natural killer (NK) cells and activated CD8$^+$ T cells, and its ligands mediates stimulatory and costimulatory signals to these cells. Here, we demonstrate that DNA-based vaccines, encoding syngeneic or allogeneic NKG2D ligands together with tumor antigens such as survivin or carcinoembryonic antigen, markedly activate both innate and adaptive antitumor immunity. Such vaccines result in highly effective, NK− and CD8$^+$ T cell-mediated protection against either breast or colon carcinoma cells in prophylactic and therapeutic settings. Notably, this protection was irrespective of the NKG2D ligand expression level of the tumor cells. Hence, this strategy has the potential to lead to widely applicable and possibly clinically useful DNA-based cancer vaccines.

NK cells | NKG2D ligands | T cells | survivin | Peyer's patches

One way to induce potent immune responses against tumors is to activate the key immune effector mechanisms. The NKG2D receptor–ligand interaction is a good candidate for such a task because it occurs at the crossroad between innate and adaptive immunity (1). NKG2D, a stimulatory lectin-like receptor, is expressed on natural killer (NK) cells, activated CD8$^+$ T cells, γδ T cells, and activated macrophages (2). It mediates costimulatory signals for CD8$^+$ T cells and stimulatory signals for NK cells and macrophages (3, 4). Ligands for NKG2D are related to class I major histocompatibility complex (MHC) molecules. In mice, NKG2D ligands include products of the retinoic acid early inducible-1 (RAE1) gene, H60 (2, 5), and UL16-binding protein-like transcript 1 (MULT1) (6). Importantly, in syngeneic mice, ectopic expression of NKG2D ligands causes NK cell-mediated rejection of transfected tumor cells (7, 8). It also primes cytotoxic T cells (CTLs), which are responsible for the rejection of subsequent challenges with tumor cells that lacked NKG2D ligands (7).

In this study, we tested our hypothesis that by engaging the NKG2D receptor, we would enhance the antitumor efficacy of DNA-based cancer vaccines by activating both innate and adaptive immunity. This task was accomplished in two different mouse model systems, a survivin-based DNA vaccine in syngeneic BALB/c mice (9) and a carcinoembryonic antigen (CEA)-based DNA vaccine in CEA-A2Kb double transgenic mice (10).

Survivin, a 16.5-kDa inhibitor of apoptosis protein, represents an almost ideal target for cancer vaccines because it is overexpressed by essentially all solid tumors and proliferating endothelial cells in the tumor vasculature. In contrast, it is poorly or only transiently expressed by normal adult tissues (11). Furthermore, overexpression of survivin in tumors is linked to decreased patient survival, increased tumor recurrence, and resistance to therapy (11), and spontaneous immune responses against survivin were recently demonstrated in various cancer patients (12). Survivin-based DNA vaccines were able to induce T cell-mediated antitumor responses without severe toxicity in mouse (9) and in clinical trials (13).

The 180-kDa oncofetal protein CEA is another appropriate vaccine target because it is overexpressed on a variety of tumors (14) but only expressed weakly on normal epithelial cells (15). This expression pattern, together with its immunogenicity, led to the development and evaluation of a variety of CEA whole gene-based or peptide-based vaccines (16, 17). In addition, techniques to measure CEA-specific CTL responses are well established (10, 18) and facilitate the verification of the specificity of immune responses induced by such vaccines.

Here, we demonstrate that engagement of the murine NKG2D receptor enhances both innate and adaptive immune responses induced by DNA-based vaccines and thereby augments their antitumor efficacies in both prophylactic and therapeutic settings.

Materials and Methods

Vector Construction and Transformation of *Salmonella typhimurium*. Expression vectors were constructed based on a pBudCE4.1 backbone (Invitrogen) as indicated. The double attenuated *S. typhimurium* (*AroA*$^-$; *dam*$^-$) strain RE88 was kindly provided by Remedyne Corporation (Santa Barbara, CA) and was transformed with DNA vaccine plasmids by electroporation as described in ref. 19.

Animals, Cell Lines, and Peptide. Female BALB/c mice, 6–8 weeks of age, were purchased from The Jackson Laboratory. C57BL/6J-CEA-A2Kb double transgenic mice were generated as described in refs. 10, 20, and 21. All animal experiments were performed according to the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*.

The murine colon carcinoma cell line MC-38-CEA-A2Kb was generated as described in refs. 10 and 20. Murine colon carcinoma cell line CT-26 was provided by I. J. Fidler (MD Anderson Cancer Center, Houston). Yac-1 cells were purchased from American Type Culture Collection (Rockville, MD), and the murine breast carcinoma cell line D2F2 was kindly supplied by W.-Z. Wei (Wayne State University, Detroit). T2, a human HLA-A2$^+$ cell line, was originally obtained from P. Cresswell (Yale University, New Haven, CT) and kindly provided by L. A. Sherman (The Scripps Research Institute). A plasmid containing the full-length murine NKG2D ligand-H60 and PE-conjugated NKG2D tetramer were generously gifted by A. Diefenbach and D. H. Raulet (University of California, Berkeley). CEA_{691} peptide (18) was kindly provided by E. Celis (Mayo Clinic, Rochester, MN) with >95% purity by HPLC.

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Abbreviations: NK, natural killer; CEA, carcinoembryonic antigen; CTL, cytotoxic T cells; DC, dendritic cells; T_{reg}, regulatory T.

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Oral Immunization and Tumor Cell Challenge. Mice were immunized twice within a 2-week interval by gavage with 100 μl of PBS containing $\approx 5 \times 10^8$ doubly mutated *S. typhimurium* harboring the expression vectors. In prophylactic models, mice were challenged with tumor cells as indicated 2 weeks after the last vaccination and in therapeutic settings 5 days before the first vaccination.

Cytotoxicity and ELISPOT Assays. Cytotoxicity was measured by a standard ^{51}Cr-release assay as described in ref. 22. ELISPOT assays were performed with an ELISPOT kit (BD Pharmingen) according to the instructions provided by the manufacturer.

Immunofluorescence. This procedure was performed on cryostat sections of Peyer's Patches fixed with acetone. Primary antibodies used were anti-mouse dendritic cell Ab, anti-mouse CD11c Ab (BD Pharmingen), recombinant mouse NKG2D/Fc Chimera (R & D Systems), and rabbit anti-human CEA Ab (DAKO). All secondary antibodies were purchased from Molecular Probes. Slides were mounted with antifade reagent (Molecular Probes) and examined with a confocal microscope (Axiovert 100TV, Zeiss).

***In Vivo* Depletions.** These depletions were performed by i.p. injection of anti-CD8 Ab 2.43 (National Cell Culture Center, Brooklyn Center, MN) at 0.6 mg per mouse or anti-CD4 Ab GK1.5 (National Cell Culture Center) at 0.4 mg per mouse 1 day before tumor cell challenge and repeated weekly, or with antiasialo GM1 Ab (Wako Chemicals, Richmond, VA) at 20 μl per mouse 1 day before tumor challenge and repeated every 5 days.

Statistical Analysis. The statistical significance of differential findings between experimental groups and controls was determined by using Student's t test. Findings were regarded as significant if two-tailed P values were <0.05.

Results

NKG2D Ligand-H60 Improves Antitumor Efficacy of DNA Vaccines Encoding Murine Survivin. Expression vectors encoding H60 (pH60), survivin (pSurv), or both (pH60/Surv) were constructed on a pBudCE4.1 (pBud) backbone (Fig. 1*A*). The expression of survivin in transfected 293T cells was confirmed by Western blot analysis (Fig. 1*B*), and the expression of H60 was confirmed by positive staining with NKG2D tetramer (Fig. 1*C*). To evaluate the vaccine-induced H60 expression *in vivo*, mice were killed 24 h after pH60 vaccination, and cryostat sections of Peyer's patches were analyzed for NKG2D ligand expression. As shown in Fig. 1*D*, NKG2D ligands (red) were detected but only at low levels in mice vaccinated with the pBud control vector. This result is not surprising, because NKG2D ligand expression can be induced by infection or cellular stress (23, 24). However, in pH60-vaccinated mice, NKG2D ligands, presumably mostly H60, are expressed at much higher levels. Notably, NKG2D expression was found inside dendritic cells (DC), proving the successful delivery of H60 to DCs. However, H60 was also expressed by other cell types, presumably macrophages (25, 26).

To test our hypothesis that NKG2D ligand-H60 can enhance the antitumor efficacy of survivin-based DNA vaccines, vaccinated syngeneic mice were challenged with murine colon carcinoma cell line CT-26. CT-26 expressed survivin (Fig. 2*A Left*) and at best expressed NKG2D ligands weakly (Fig. 2*A Right*). Tumor cells with low or negative NKG2D ligand expression had been reported to be more tumorigenic (7). Likewise, as shown in our experiment, tumors colonized in the lungs of all mice treated with PBS or pBud 25 days after challenge (Fig. 2*B*). Importantly, only a DNA vaccine encoding both a NKG2D ligand (H60) and survivin effectively protected the mice against tumor challenge (Fig. 2*B*).

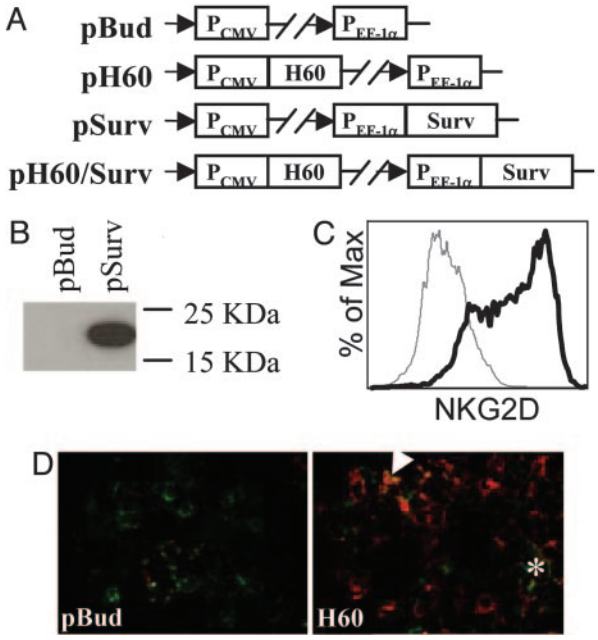

Fig. 1. Construction and expression of vectors encoding murine NKG2D ligand-H60 and survivin. (*A*) Expression vectors were constructed based on the pBudCE4.1 plasmid backbone. (*B*) Expression of murine survivin was detected by Western blotting analyses of pSurv-transfected 293T cells, by using rabbit anti-mouse survivin antibody. (*C*) Expression of H60 was demonstrated by flow cytometry using NKG2D tetramer. 293T cells were transfected with either pH60 (thick black line) or pBud vectors (thin gray line). (*D*) Expression of NKG2D ligands in Peyer's patches *in vivo*. Mice were vaccinated with either pBud (*Left*) or pH60 (*Right*). Green, DC Ab; red, NKG2D/Fc chimera; arrowhead, colocalization; *, both markers expressed on the same cells without colocalization.

In vivo depletions were performed to determine which cell population was responsible for this protection. Tumors developed so rapidly in $CD8^+$ or NK cell depletion mice that the mice had to be killed 2 days earlier (Fig. 2*C*), suggesting that these effector cells are involved in the normal surveillance against CT-26 tumor cells. Moreover, these depletions abrogated most of the tumor protective effect induced by the pH60/Surv vaccine (Fig. 2*C*), indicating that the protection was mainly mediated by NK and CD8 T cells. In contrast, the depletion of CD4 cells from immune-competent mice protected against tumor cell challenge in both pBud and pH60/Surv groups (Fig. 2*C*), suggesting that $CD4^+$ T cells contribute mainly to the negative regulation of antitumor immunity in our experimental system.

The DNA vaccines were also tested in therapeutic settings. Experimental pulmonary metastases were induced by i.v. injection of 1×10^5 CT-26 cells, which previously resulted in tumor colonization to lungs within 3 days (27). Mice were then vaccinated. Significantly, in each of two similar experiments, 75% of mice treated with the pH60/Surv vaccine survived (Fig. 2*D*), with most of them showing no or minimal tumor metastases (data not shown). In comparison, only 10% of mice survived in the empty pBud vector control group, all of them with >50% of their lung surface covered by fused tumor metastases (data not shown). The pSurv or pH60 vaccines failed to protect mice significantly.

Because survivin is overexpressed by essentially all solid tumor cells (11), the pH60/Surv vaccine should be effective against tumors of different origin. To test this contention, a breast cancer cell line, D2F2, was used for tumor cell challenge. D2F2

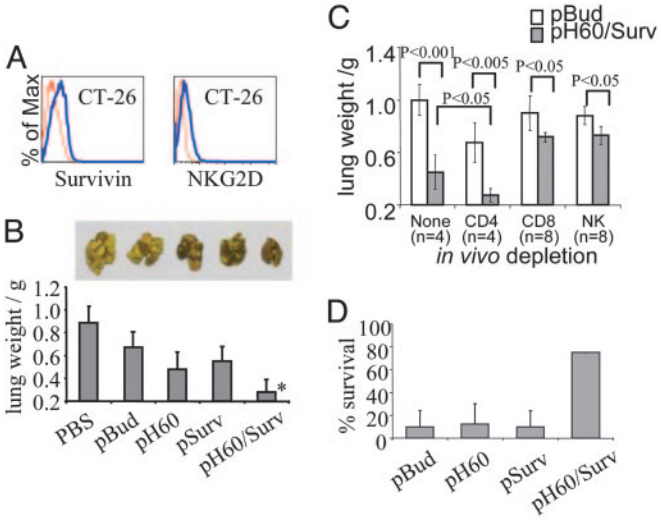

Fig. 2. NKG2D ligand H60 enhances the antitumor efficacy of survivin-based DNA vaccines in a murine CT-26 colon carcinoma model. (*A*) Endogenous expression (thick blue lines) in CT-26 cells of survivin (*Left*) or NKG2D ligand (*Right*). Thin red lines, staining controls. (*B*) Prophylactic setting. Vaccinated mice ($n = 8$) were challenged i.v. with 1×10^5 CT-26 colon carcinoma cells. (*Upper*) Representative lungs. (*Lower*) Average lung weight. Normal lung weight is ≈0.2 g. ✻, $P < 0.00005$, 0.001, 0.02, or 0.005 compared with PBS, pBud, pH60, or pSurv, respectively. Experiments were repeated 3 times with similar results. (*C*) *In vivo* depletion assays. Experiments were terminated 2 days earlier in CD8- and NK-depleted mice than in control and CD4-depleted animals. (*D*) Therapeutic setting. Mice were challenged i.v. with 1×10^5 CT-26 cells and later vaccinated. The results presented are the average of two separate experiments ($n = 12$ and 4).

cells expressed survivin; they also expressed NKG2D ligands at an intermediate level (Fig. 3*A*). In the prophylactic setting (Fig. 3*B*), 12.5% of the mice were free of tumors in the pBud control group 30 days after tumor challenge, in agreement with a previous report on partial rejection of tumors expressing NKG2D ligands at intermediate levels (7). The number of tumor-free animals improved to 25% and 62.5% in pH60 or pSurv vaccinated groups of mice, respectively. Most importantly,

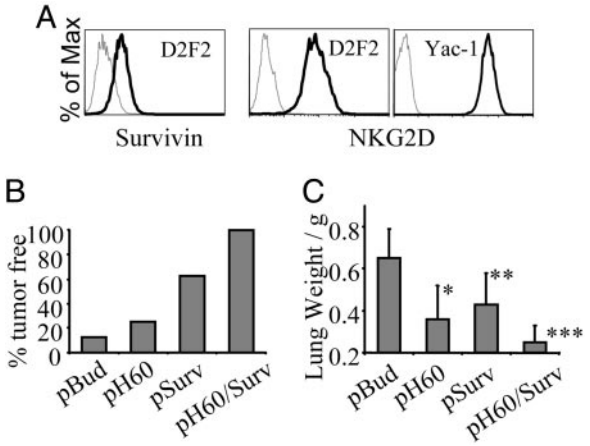

Fig. 3. The pH60/Surv vaccine protects mice in a murine D2F2 breast carcinoma model. (*A*) Endogenous expression of survivin (*Left*) and NKG2D ligands (*Center*) by D2F2 cells (thick black lines), compared with NKG2D ligand positive control-Yac-1 cells (*Right*). Thin gray lines, staining controls. (*B*) Prophylactic setting. (*C*) Therapeutic setting. ✻, $P < 0.01$; ✻✻, $P < 0.05$ (compared with pBud); ✻✻✻, $P < 0.0002$ and 0.05 (compared with pBud and pSurv, respectively).

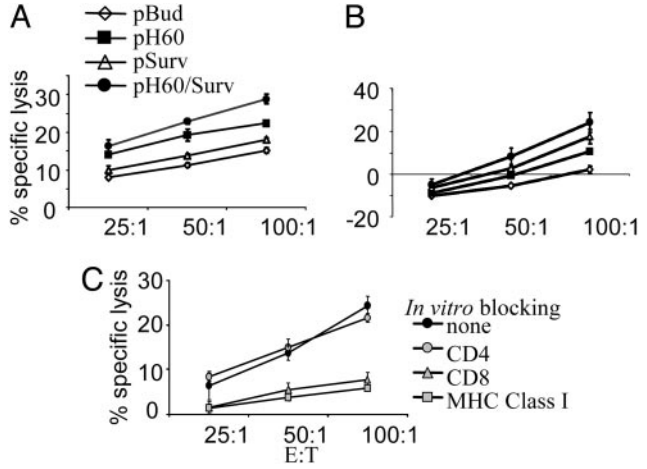

Fig. 4. The pH60/Surv enhanced NK and $CD8^+$ T cell activity. Freshly isolated or *in vitro*-stimulated splenocytes were tested in a standard ^{51}Cr-release assay against Yac-1 (*A*) or CT-26 (*B*) target cells. ◇, pBud group; ■, pH60 group; △, pSurv group; ●, pH60/Surv group. Experiments were repeated twice with similar results. (*C*) Cytotoxicity assays against CT-26 target cells in the absence or presence of blocking Abs. *In vitro*-stimulated splenocytes were originally isolated from pH60/Surv-vaccinated mice. ●, no blocking Ab; shaded symbols, specific lysis in the presence of anti-CD4 (○), anti-CD8 (△), or anti MHC class I (□) Abs. Experiments were repeated once with similar results.

only in the pH60/Surv group were 100% of mice completely free of tumors (Fig. 3*B*). In a therapeutic setting, a higher dose of D2F2 cells (3×10^5) was used to challenge mice and resulted in tumor development in all mice within 21 days in pBud group. In this case, vaccination with pH60/Surv induced the best therapeutic effect (Fig. 3*C*). Taken together, these data suggest that the pH60/Surv vaccine is optimally effective in both prophylactic and therapeutic settings against tumors of different origin, irrespective of their NKG2D ligand expression levels.

The pH60/Surv Vaccine Activates both Innate and Adaptive Immune Responses. The known distribution and functions of NKG2D receptors (3, 4) and the results from NK and CD8 depletion experiments prompted us to directly assess whether coexpression of NKG2D ligand-H60 in the survivin-based DNA vaccine leads to enhanced NK- and CTL-mediated tumor cell killings. In fact, NK target cell killing was significantly enhanced in mice immunized with the pH60 vaccine and was further improved in pH60/Surv-vaccinated mice (Fig. 4*A*). Splenocytes isolated from pH60/Surv-vaccinated mice also exhibited the highest cytotoxicity against CT-26 target cells (Fig. 4*B*). This cytotoxicity was largely inhibited by Abs against CD8 or MHC class I molecules but not affected at all by anti-CD4 Ab (Fig. 4*C*), suggesting that the cytotoxicity detected was mediated mainly by MHC class I-restricted $CD8^+$ T cells.

To verify that the improved vaccine efficacy was indeed attributable to ligation of NKG2D receptor, another NKG2D ligand, RAE1, was also included in the vaccine, replacing H60. The expression of RAE was confirmed by flow cytometry (Fig. 5*A*). This pRAE1/Surv vaccine also proved superior in protecting mice from CT-26 challenge (Fig. 5*B*) and inducing NK (Fig. 5*C*) and CTL (Fig. 5*D*) responses . These data confirm that the combination vaccines enhance antitumor immunity by engaging the NKG2D receptor, suggesting a strong adjuvant effect of NKG2D ligands for the survivin-based DNA vaccine.

NKG2D Ligand H60 Enhances the Efficacy of CEA-Based DNA Vaccines. The difficulty in finding survivin-negative tumor cells prevented us from further demonstrating the specificity of the immune

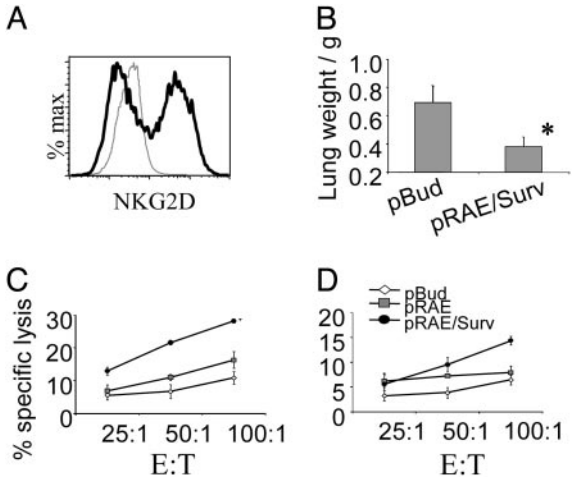

Fig. 5. Another NKG2D ligand, RAE1, enhances the antitumor efficacy of survivin-based DNA vaccines. (*A*) Expression of RAE1 was demonstrated by flow cytometry using NKG2D tetramer. COS-7 cells were transfected with the pRAE (thick black line) or pBud control (thin gray line) vector. (*B*) *In vivo* CT-26 colon carcinoma model. Vaccinated mice ($n = 8$) were challenged i.v. with 1×10^5 CT-26 cells. ✻, $P < 0.02$ compared with pBud control group. Freshly isolated (*C*) or *in vitro*-stimulated (*D*) splenocytes were tested in a standard ^{51}Cr-release assay against Yac-1 (*C*) or CT-26 (*D*), respectively. ◇, pBud group; □, pRAE group; ●, pRAE/Surv group.

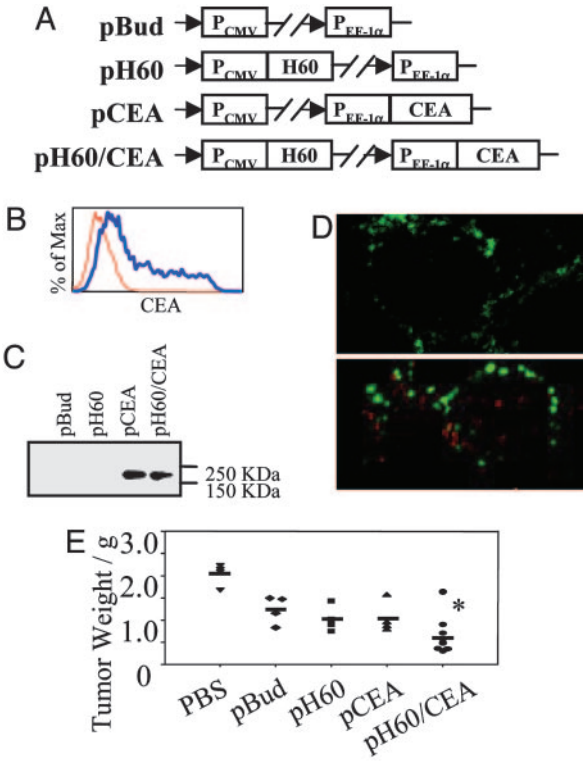

Fig. 6. NKG2D ligand H60 enhances the antitumor efficacy of CEA-based DNA vaccines. (*A*) Expression vectors were constructed based on the pBudCE4.1 backbone. (*B*) Flow cytometric analyses of 293T cells transfected with pCEA (thick blue line) or the empty pBud (thin red line) vector by using anti-CEA Ab. (*C*) Western blotting analyses of 293T cells transfected with different expression vectors by using anti-CEA Ab. (*D*) *In vivo* expression of CEA (red) in the Peyer's patches from pBud-vaccinated (*Upper*) or pCEA-vaccinated (*Lower*) mice. Each panel indicates two representative CD11c$^+$ (green) dendritic cells. (*E*) Vaccinated CEA-A2Kb double transgenic mice were challenged s.c. with 6×10^5 MC-38-CEA-A2Kb cells and killed 28 days later. Tumor weights from each mouse are represented by individual symbols. Solid line, average tumor weights of each group. ✻, $P < 0.0002$ or 0.05 (compared with PBS or pBud control groups, respectively). Experiments were repeated three times with similar results.

response. Consequently, we took advantage of our CEA-based DNA vaccine (16). We had already identified a specific HLA-A2-restricted CTL epitope in CEA-A2Kb double transgenic mice (10). Because H60 is not expressed in C57BL/6 mice (28), this approach was also selected to assess whether an allogeneic NKG2D ligand could enhance the efficacy of DNA-based vaccines and the feasibility for clinical applications. The corresponding DNA vaccine constructs are depicted in Fig. 6*A*, and the CEA expression of transfected 293T cells was confirmed by positive staining in both flow cytometric (Fig. 6*B*) and Western blot (Fig. 6*C*) analyses. Vaccine-induced CEA expression *in vivo* was further evaluated with cryostat sections of Peyer's patches from vaccinated mice. CEA expression was shown to localize to CD11c$^+$ cells (Fig. 6*D Lower*), whereas control mice did not reveal any CEA expression in the Peyer's patches (Fig. 6*D Upper*).

Vaccine efficacy was further evaluated by a series of *in vivo* studies. CEA-A2Kb double transgenic mice were used because they are peripherally tolerant to CEA and express HLA-A2, enabling us to check vaccine efficacy and immune response against HLA-A2-restricted epitopes. Immunization with the pH60/CEA DNA vaccine effectively protected these transgenic mice from challenges of colon carcinoma cells MC-38 transfected with CEA and A2Kb (MC-38-CEA-A2Kb, Fig. 6*E*) but not from the parental MC-38 cells (data not shown). These findings demonstrate the specificity of the protection and suggest that the NKG2D ligand H60 also enhances the antitumor efficacy of CEA-based DNA vaccines.

Vaccination with pH60/CEA Enhances HLA-A2-Restricted, CEA_{691}-Specific CTL Responses. As expected, splenocytes from pH60/CEA-vaccinated mice not only exhibited the highest killing against NK target cells (Fig. 7*A*) but also showed the most effective cytotoxicity against MC-38-CEA-A2Kb target cells (Fig. 7*B*) and not the parental MC-38 cells (Fig. 7*C*).

Next, we focused on the specific responses against one of the HLA-A2-restricted CTL epitopes, CEA_{691} (18). The pH60/CEA-vaccinated group displayed significant killing of CEA_{691}-loaded HLA-A2$^+$ T2 cells but not unloaded T2 cells (Fig. 7*D*). To further substantiate this finding, ELISPOT assays were performed with splenocytes freshly isolated from vaccinated CEA-A2Kb double transgenic mice. CEA_{691}-specific, IFN-γ-secreting T cells were already present in pCEA-vaccinated mice; however, this reactivity was greatly enhanced in mice immunized with the pH60/CEA vaccine (Fig. 7*E*). Consequently, we demonstrated that the immune response induced by the pH60/CEA vaccine was still CEA-specific but had attained a higher level of efficacy.

Discussion

T cells targeting tumor-associated antigens are readily detectable in cancer patients and in those patients that received cancer vaccines, yet in most cases, such T cells fail to eradicate the tumor *in vivo*. Thus, it is clear that established tumors can induce immune tolerance through not well defined mechanisms (29). Consequently, we assumed that immunization strategies that employ different arms of the immune system should overcome such tolerance.

In our studies, DNA vaccines encoding tumor-associated antigens and the NKG2D ligands were transformed into double attenuated *S. typhimurium* (*Dam*$^-$; *AroA*$^-$), which target these genes to secondary lymphoid organs, i.e., Peyer's patches (19, 25). There, the bacteria can be phagocytosed by antigen-

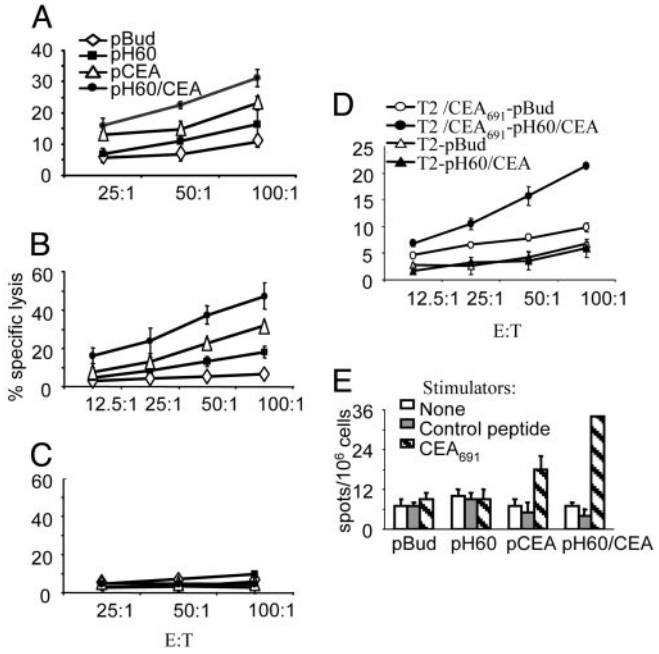

Fig. 7. The pH60/CEA vaccine enhanced NK- and CEA-specific CTL responses. (*A*) Standard ^{51}Cr release assays were performed with freshly isolated splenocytes against Yac-1 NK target cells. *In vitro*-stimulated splenocytes were tested in cytotoxicity assays against MC-38-CEA-A2Kb (*B*) or MC-38 (*C*) target cells. (*A–C*) ◇, pBud group; ■, pH60 group; △, pCEA group; ●, pH60/CEA group. (*D*) Cytotoxicity of *in vitro*-stimulated splenocytes from pBud (open symbols) or pH60/CEA (closed symbols) groups against unloaded (△) or CEA_{691}-loaded (○) T2 target cells. (*E*) ELISPOT assays were performed with freshly isolated splenocytes either without (open bars) or in the presence of control peptide (kindly provided by J. Schlom of the National Institutes of Health; shaded bars) or CEA_{691} peptides (striped bars) at 10 μg/ml.

presenting cells such as DCs. In addition, *Salmonella* provide ligands for toll-like receptors or "danger signals," which can induce DC activation and maturation. The genes encoded by vectors can be then transcribed, translated, expressed, and processed by DCs (30). Here, in fact, we demonstrate the *in vivo* expression of H60 or CEA encoded by our DNA vaccine in DCs located inside Peyer's patches, proving the efficiency of this delivery system.

DCs are known to support the tumoricidal activity of NK cells (31). In our vaccine setting, the interactions of NKG2D ligands expressed on DCs with the NKG2D receptor expressed on NK cells can directly activate these effector cells as reported in ref. 3. In addition cytokines secreted by activated DCs are likely to contribute to the robust NK activity detected in our pH60/Surv- or pH60/CEA-vaccinated mice.

DCs are the most proficient antigen-presenting cells (32) and have extraordinary capacity to activate naïve T cells (33). Once specific peptide/MHC complexes presented by DCs are recognized by the T cell receptor complex on $CD8^+$ T cells, costimulatory signals provided by NKG2D–ligand interactions between these two populations will contribute to more effective T cell priming. This approach induced effective T cell responses, both in pH60/Surv-vaccinated syngeneic BALB/c mice and in pH60/CEA-vaccinated CEA-A2Kb double transgenic mice.

DCs, NK cells, and T cells are not independent in their functions but are subject to intense crosstalks. By secreting cytokines such as IFN-γ and TNF-α, activated NK cells can induce the maturation of DCs, which become stable and resistant to tumor-related suppressive factors, and show a strongly enhanced ability to induce Th1 and CTL responses (34). This interaction may contribute to the enhanced CTL killing observed in the pH60 (Figs. 4*B* and 7*B*) or pRAE (Fig. 5*D*) groups compared with control mice. $CD8^+$ T cells are shown to induce maturation of DCs in the absence of $CD4^+$ cells and CD40 ligation (35). By secreting IFN-γ, activated but not resting $CD8^+$ T cells can induce differentiation of monocytes into DCs and restore the stimulatory capacity of IL-10-treated antigen-presenting cells (36). The secretion of IL-10 by tumor cells has been shown to be one of the mechanisms tumors use to escape immune surveillance (37). This CTL feedback can explain the enhanced NK killing of pSurv (Fig. 4*A*) or pCEA group (Fig. 7*A*) compared with the control group.

Besides the aforementioned, activated NK cells and CTLs can also enforce each other's activation directly. Activated NK cells boost the ongoing adaptive responses by producing IFN-γ, which promotes the Th1 polarization of antigen-specific T cells (38). Antigen-specific T cells can directly activate NK cells by secreting IL-2 (39), which can also provide signals required by NK cells in "helping" DCs (40). Thus, by delivering our vaccines with *Salmonella*, engaging NKG2D receptor, and providing tumor-associated antigens, our DNA vaccine can efficiently activate DCs, NK cells, and CTLs, presumably in Peyer's patches. The crosstalk between these different populations can reinforce their activation, giving the immune system the edge to overcome tumor-induced immune-suppression. These effects may be translated into the tumor protection induced by our vaccine in both prophylactic and therapeutic models.

Recently, regulatory T (T_{reg}) cells, particularly $CD4^+CD25^+$ T_{reg} cells, have reached the spotlight in tumor immunology. Depletion of $CD4^+CD25^+$ T cells in mice improves tumor clearance (41) and enhances the response to immune-based therapy (42). Tumor infiltrating T_{reg} cells have been clinically associated with reduced survival (43). In our system, $CD4^+$ T cells, presumably mediated by T_{reg} cells, mainly contributed to the negative regulation of immune responses because their depletion led to better protection against tumor challenges. T_{reg} cells, as all $CD4^+$ T cells, do not express NKG2D receptor even after their activation (3). By engaging this receptor, our vaccine can achieve preferential activation of NK and $CD8^+$ T cells and, thus, may tilt the balance toward immune surveillance and breakage of T_{reg}-mediated peripheral tolerances to tumor-associated antigens. However, it is also possible that some $CD4^+$ T cell subpopulations are required for optimal immune responses and generation of effective immune response and memory as reported in refs. 44 and 45.

We demonstrated here that our pH60/Surv vaccine effectively protected mice against two different tumors: CT-26 colon carcinoma cells, which express very low or negative levels of NKG2D ligands, and D2F2 breast carcinoma cells, which express NKG2D ligands at an intermediate level. However, the pH60/Surv vaccine worked in both models almost equally well, suggesting the potential utility of this vaccine for a variety of tumors, despite their varying NKG2D ligand expression levels. In addition, utilization of another NKG2D ligand, RAE1, in similar vaccines induced protection against tumor challenge, proving that the engagement of the NKG2D receptor is indeed responsible for the improved antitumor efficacy of such vaccines.

However, it is well known that NKG2D ligands display a great degree of polymorphism both in mice (46) and humans (47). Thus, to develop a widely applicable cancer vaccine, it should be determined whether an allogeneic NKG2D ligand can also serve as an adjuvant for DNA vaccines. Such an evaluation is facilitated by the fact that H60 is not expressed in C57BL/6 mice (28) and that a $H\text{-}2K^b$-restricted CTL response was reported to mediate graft-vs.-host disease (48). In this regard, in our pH60/CEA vaccine, H60 may provide an allogeneic stimulus as well as the stimulation and costimulation of NK and T cells. It is possible that the anti-H60 immune response is so dominant (49) that it overshadows the tumor-specific responses. However, our data

indicate that the pH60/CEA vaccine successfully induced both potent NK- and CEA-specific CTL responses. These data suggest that allogeneic NKG2D ligands are also effective adjuvants and, thus, applicable to a variety of DNA-based cancer vaccines. Furthermore, the fact that such ligands enhance immune responses against a human tumor antigen like CEA suggests their potential for clinical applications. It is of course possible, given the NKG2D expression pattern and other differences between human and mouse (24), that the exact same strategy may not work with the same efficacy in humans. However, sufficient employment of both innate and adaptive arms of the immune system may still be a sensible way to overcome the hurdle created by the tumors.

In summary, we critically evaluated NKG2D ligands as adjuvants for DNA-based cancer vaccines. We demonstrated here that coexpression of NKG2D ligands in DNA-based cancer vaccines effectively enhances their antitumor efficacy by activating both innate and adaptive immune responses. Such DNA vaccines are well suited to eradicate residual tumor cells and to possibly boost an immune response against cancer relapse, thus making them relevant for future clinical applications. This contention is supported by the impressive antitumor effect achieved by our pH60/Surv DNA vaccine in a therapeutic setting featuring established metastases of murine breast and colon carcinoma.

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A DNA-based cancer vaccine enhances lymphocyte crosstalk by engaging the NKG2D receptor

Running Title: Engaging NKG2D receptor enhances crosstalk

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Key Words: NKG2D ligands; innate and adaptive immunity; Peyer's Patches; crosstalk.

Abbreviation: DCs, dendritic cells; NK, natural killer; CTL, cytotoxic T cells; TAA, tumor associated antigen; PSGL-1, P-selectin glycoprotein-1.

Abstract

The NKG2D receptor is a stimulatory receptor expressed on NK cells and activated CD8 T cells. We previously demonstrated that engaging the NKG2D receptor markedly improved the efficacy of a survivin-based DNA vaccine. The combination vaccine encoding both the NKG2D ligand H60 and survivin activates both innate and adaptive antitumor immunity, resulting in better protection against tumors of different origin and NKG2D expression levels. Here we demonstrate that the enhanced vaccine efficacy is in part attributable to increased crosstalk between lymphocytes. Depletion of CD8 T cells during priming reduces the vaccine-induced activation of dendritic cells (DCs) and NK activity. Depletion of NK cells during priming leads to reduced DC activation and CTL activity. However, depletion of CD4 T cells results in activation of DCs, NK and CD8 T cells and enhances NK cell activity. The pH60/Survivin vaccine also increases DC and NK cells, but decreases CD4 T cells homing to Peyer's patches, presumably as a result of changes in the their homing receptor profile. Thus, by preferentially activating and attracting positive regulators, and reducing negative regulators in Peyer's patches, this dual function DNA vaccine induces a microenvironment more suitable for NK cell activation and T cell priming.

Introduction

T cells which target tumor associated Ag (TAA) are readily detectable in cancer patients, including those who received cancer vaccines. However, in most cases such T cells fail to eradicate tumors in these patients. Thus, it is apparent that established tumors can induce immune tolerance through yet poorly-defined mechanisms.[1] We hypothesized that immunization strategies which employ different arms of the immune system could overcome this immune tolerance. The NKG2D receptor-ligand interaction is a good candidate to test this hypothesis since it occurs at the crossroad between innate and adaptive immunity.[2] NKG2D, a stimulatory lectin-like receptor, is expressed on natural killer (NK) cells, activated $CD8^+$ T cells, γδ T cells and activated macrophages.[3] It mediates co-stimulatory signals for $CD8^+$ T cells and stimulatory signals for NK cells and macrophages.[4,5] NKG2D ligands are related to class I major histocompatibility complex (MHC) molecules. In mice, such ligands include products of the retinoic acid early inducible –1 (RAE1) gene, H60,[3,6] and the UL16-binding protein-like transcript 1 (MULT1).[7] Importantly, in syngeneic mice, ectopic expression of NKG2D ligands causes NK cell-mediated rejection of transfected tumor cells.[8,9] It also primes cytotoxic T cells (CTLs) which are responsible for rejection of subsequent challenges with tumor cells lacking NKG2D ligand expression.[8] In our prior study, we demonstrated that engagement of the murine NKG2D receptor (by using a DNA vaccine encoding one of its ligands H60) enhanced both innate and adaptive immune responses induced by a survivin-based DNA vaccine. This, in turn, augmented the vaccine's antitumor efficacy in both prophylactic and therapeutic settings against tumors of different origin and NKG2D expression levels.[10]

Survivin, a 16.5 kDa inhibitor of apoptosis protein, represents an almost ideal target for cancer vaccines since it is overexpressed by essentially all solid tumor cells as well as by proliferating endothelial cells in the tumor microenvironment. In contrast, survivin is poorly, or only transiently expressed by normal adult tissues.[11] Furthermore, over-expression of survivin in tumors is linked to decreased patient survival, increased tumor recurrence and resistance to therapy.[11] In addition, spontaneous immune responses against survivin were recently demonstrated in a variety of cancer patients.[12] Survivin-based DNA vaccines were also shown to induce T cell-mediated antitumor responses without severe toxicities in pre-clinical[13] and clinical trials.[14]

Here, we demonstrate that the enhanced vaccine efficacy of NKG2D ligand H60 plus survivin is in part due to increased lymphocyte crosstalk, thus proving that activation of both the innate and adaptive arms of the immune system provides an attractive strategy to overcome tumor-induced peripheral immune tolerance.

Materials and Methods

Animals and cell lines

Female BALB/c mice, 6–8 wk of age, were purchased from the Jackson Laboratory (Bar Harbor, ME). All animal experiments were performed according to the National Institutes of Health Guides for the Care and Use of Laboratory Animals.

The murine colon carcinoma cell line CT-26 was kindly provided by Dr. I. J. Fidler (MD Anderson Cancer Center, Houston, TX). Yac-1 cells were purchased from the American Type Culture Collection (ATCC, Rockville, MD). A plasmid containing the full-length murine NKG2D ligand-H60 was a generous gift from Drs. A. Diefenbach and D. H. Raulet (University of California, Berkeley, CA).

Oral immunization

The double attenuated *Salmonella typhimurium (AroA$^-$; dam$^-$)* strain RE88 was kindly provided by Remedyne Corporation (Santa Barbara, CA). Expression vectors were constructed based on a pBudCE4.1 backbone (Invitrogen, Carlsbad, CA) and transformed into *Salmonella typhimurium* as previously described.[10] Mice were immunized twice at a 2-wk interval by gavage with 100 μl 5% sodium bicarbonate containing approximately $5x10^8$ doubly mutated *S. typhimurium* harboring the expression vectors.

In vivo depletions

CD8 or CD4 depletions were performed by i.p. injection of anti-CD8 Ab 2.43 or anti-CD4 Ab GK1.5 (both obtained from the National Cell Culture Center, Brooklyn Center, MN) at 0.5 mg /mouse, starting 1 d prior to the first vaccination (priming phase) or 3 d after the last vaccination (effector phase) and repeated weekly. NK depletions were achieved by i.p. injection of anti asialo GM1 Ab (Wako Chemicals USA, Inc., Richmond,

VA) at 20 μl /mouse, starting on 1 d prior to or 3 d post vaccination and repeated every 5 d.

Cytotoxicity and flow cytometry

Cytotoxicity was measured by a standard ^{51}Cr-release assay as previously described.[15] Flow cytometry was performed on lymphocytes isolated from Peyer's Patches or other lymphoid tissues 1 d post vaccination with a BD LSRII cell sorter (Becton Dickinson, San Jose, CA), equipped with DIVA software. Data were analyzed with FlowJo software (Tree Star, Inc, Stanford, CA). All Abs were obtained from BD Pharmingen (San Diego, CA), except for Abs against NKG2D and GITR, which were purchased from eBioscience (San Diego, CA).

Inhibition of CD3-induced splenocyte proliferation by $CD4^+CD25^+$ cells

$CD3^+CD4^+CD25^+$ cells were purified by flow cytometric cell sorting from splenocytes isolated from control or pH60/Surv-vaccinated mice 2 wks after the last vaccination. Splenocytes from normal mice were labeled with 5μM CFSE and incubated for 3 d with 10μg/ml anti-CD3 Ab in the absence or presence of purified $CD3^+CD4^+CD25^+$ cells at a ratio of 100:1. These cells were then harvested and analyzed for CFSE intensity by flow cytometry.

Statistical Analysis

The statistical significance of differential findings between experimental groups and controls was determined by Student's *t* test. Findings were regarded as significant, if two-tailed *P* values were <0.05.

Results

The H60-Survivin vaccine (pH60/Surv) induces activation of DCs, NK and T cells in Peyer's patches

We demonstrated previously that a DNA vaccine encoding both a murine NKG2D ligand-H60 and the TAA survivin protected mice from lethal tumor cell challenges in both prophylactic and therapeutic settings. These anti-tumor effects were the result of enhanced NK and CTL activities.[10]

To further elucidate the mechanisms involved in this tumor protection, we now focused on Peyer's patches based on the fact that these secondary lymphoid organs were shown to be the site of antigen (Ag) expression[10] and priming for DNA vaccines carried by attenuated *Salmonella typhimurium*.[16,17] Vaccine-induced cell activations were analyzed by 6-color flow cytometry. The most striking observation was the activation state of DCs, NK and CD8 T cells in Peyer's patches following pH60/Surv vaccination. Activation was demonstrated by upregulation of CD80 and CD86 on DCs (Fig 1A), as well as CD69 and NKG2D receptor on NK cells (Fig. 1B) and CD8 T cells (Fig 1C). CD4 T cells also revealed a slight up-regulation of CD69 (data not shown). Cell activation induced by DNA vaccines encoding only H60 or Survivin was either not detectable or minimal compared to the pBud control group (Figure 1A-C). These data suggest that the dual-function vaccine induces synergistic responses inside Peyer's patches.

Cell activation was also investigated by analyzing lymphocytes isolated from spleens, peripheral blood, inguinal, axillary and brachial lymph nodes. These cells, isolated from pH60/Surv vaccinated mice, either revealed a profile similar to that of the

control group or showed a slight upregulation of activation markers (data not shown). These findings, as well as our previous data demonstrating a vaccine-induced Ag and H60 expression inside Peyer's patches,[10] suggested that the microenvironment inside Peyer's patches is indeed the location for the specific immune stimulation initiated by the pH60/Surv vaccine.

CD8 T and NK cells are positive contributors while CD4 T cells mainly mediate negative regulation in pH60/Surv-induced immune responses

In vivo cell depletion experiments were performed to test the role played by each cell population in synergistic responses. Depletion of CD8 cells reduced the pH60/Surv-induced activation of DCs (Fig 2A). More importantly, the depletion of CD8 T cells prior to vaccination, i.e. the priming phase, but not after vaccination, i.e. the effector phase, resulted in reduced killing of Yac-1 NK-target cells at effector to target ratios of 50:1 and 100:1 (Fig 2B). The cytotoxicity against Yac-1 cells is largely dependent on NK cells since their depletion *in vivo* resulted in an over 80% reduction of this activity (Fig. 2C). The CD8 depletion-induced reduction in NK activity is not the result of a decrease in NK cell percentages, since CD8 depletion did not significantly affect the percentage NK cells in the spleen (Fig. 2B upper panel). Taken together, these data suggest that CD8 T cells are positive contributors to lymphocyte crosstalk and enhance vaccine-induced DC activation and NK activity.

Similarly, depletion of NK cells also led to reduced pH60/Surv-induced DC activation (Fig 3A). NK cell depletion during the priming phase, but not the effector phase, resulted in decreased cytotoxicity against CT-26 colon carcinoma cells (Fig 3B). This killing was previously shown to be mediated by CD8 T cells,[10] and *in vivo* depletion

of these cells resulted in more than 70% reduction of such activity (Fig. 3C). Since NK depletion in priming or effector phase induced similar changes in CD8 distribution in the spleen (Fig. 3B upper panel), these data indicate the positive role of NK cells in the crosstalk between lymphocytes induced by the pH60/Surv vaccine.

Mice depleted of CD4 cells, prior to pH60/Surv vaccination, displayed activation of DCs, CD8 T and NK cells inside Peyer's patches (Fig 4A). Similar changes were also observed for cells isolated from CD4-depleted pBud control mice (data not shown). These findings are in agreement with our prior notion that CD4 T cells function as negative regulators during immune surveillance and immune responses induced by pH60/Surv.[10] The depletion of CD4 T cells during the priming phase led to enhanced killing of NK target cells, whereas depletion of CD4 T cells during the effector phase did not contribute significantly (Fig 4B). Since the depletion of CD4 T cells leads to similar changes in lymphocyte distribution in the spleen (Fig. 4B, upper panel), these data suggest a negative regulatory role for CD4 T cells in vaccine induced immune responses. Surprisingly, the *in vivo* depletion of CD4 T cells during both priming and effector phases resulted in a similar reduction in CT-26 tumor cell killing (Fig 4C).

The pH60/Surv vaccine induces differences in lymphocyte homing to Peyer's patches

Activation of lymphocytes also leads to changes in their homing receptors.[18-21] The extensive lymphocyte activation induced by the pH60/Surv vaccine prompted us to look at the expression of their homing receptors. As anticipated, both CD4 and CD8 T cells down-regulated L-selectin (CD62L, Fig. 5A). However, these cells revealed different changes in the expression of other adhesion molecules (Fig. 5A). CD4 T cells showed a

slight up-regulation in the functional P-selectin glycoprotein ligand-1 (PSGL-1) and in $\alpha4\beta7$ integrin, whereas CD8 T cells upregulated functional PSGL-1, CCR7 and α_E integrin (CD103) more significantly. NK cells revealed changes similar to those of CD8 T cells (data not shown). These differences in homing receptor expression may translate to changes in the distribution of lymphocyte populations in Peyer's patches. In fact, when compared to the control group, the pH60/Surv vaccinated group of mice displayed an increase in the percentage of DCs and NK cells, and a decrease in CD4 T cells, with no significant changes in the percentage of CD8 T cells (Fig. 5B). Taken together, these data suggest that the pH60/Surv vaccine preferentially attracts and activates positive regulators in Peyer's patches and initiates an increase in positive crosstalk during the priming phase.

The pH60/Surv vaccine decreases negative regulation mediated by $CD4^+CD25^+$ regulatory T cells (Treg) in Peyer's patches

The negative regulatory effect of CD4 T cells prompted us to focus on $CD4^+CD25^+$ T cells, since Treg cells with this phenotype were demonstrated to limit immune response to ensure immune tolerance.[22] The pH60/Surv vaccine induced a decrease in the percentage of these $CD4^+CD25^+$ T cells in Peyer's patches as compared to the control group (Fig 6A). These $CD4^+CD25^+$ T cells showed no enhanced activation, as indicated by their CD25, CD69 and glucocorticoid-induced TNFR-related gene (GITR) expression levels (Fig. 6B). GITR is expressed constitutively at high levels by Treg cells and is further upregulated upon activation.[23,24] Functionally, highly purified $CD4^+CD25^+$ cells (over 99% purity) from pH60/Surv-vaccinated mice showed an inhibitory capacity comparable to that of $CD4^+CD25^+$ cells of the pBud control group (Fig. 6C). These data

demonstrate that the pH60/Surv vaccine decreased the number of Treg cells in Peyer's patches while maintaining similar activity levels, thus resulting in an overall decrease in negative regulation.

The pH60/Surv vaccine induced long-term T cell memory

We then tested the hypothesis that increased lymphocyte crosstalk leads to better T cell priming. One way to test effective T cell priming is to focus on the establishment of immune memory. In this regard, mice were challenged i.v. with $1x10^5$ CT-26 colon carcinoma cells as late as 5 months after the last vaccination. The pH60/Surv group of mice revealed reduced tumor burden (Fig 7A) and sustained high CTL activity when compared with the control group (Fig 7B). These findings demonstrate the establishment of long-lived memory T cells in pH60/Surv-vaccinated mice.

Discussion

DCs, NK and T cells are not independent in their function but are subject to intense crosstalk. We demonstrated in our experiments this crosstalk by *in vivo* depletion assays. The depletion of NK or CD8 T cells led to a decrease in DC activation, thereby demonstrating the positive contribution of NK and CD8 T cells to the activation of DCs *in vivo*. Several other studies have also confirmed this "helper" function of NK cells and CD8 T cells. For instance, by secreting cytokines such as IFN-γ and TNFα, activated NK cells were shown to induce the maturation of DCs, which became resistant to tumor-related suppressor factors and showed a strongly enhanced ability to induce Th1 and CTL responses.[25] Similarly, $CD8^+$ T cells were also reported to induce maturation of DCs in the absence of $CD4^+$ T cells and CD40 ligation.[26] Furthermore, by secreting IFN-γ, activated $CD8^+$ T cells could induce the differentiation of monocytes into DCs and restored the stimulatory capacity of IL-10-treated APCs.[27] The importance of this latter finding is that the secretion of IL-10 by tumor cells has been shown to be one of the mechanisms by which tumor cells escape immune surveillance.[28]

In addition to the ability of NK and CD8 T cells to regulate DC activation, they also regulate the activity of each other. We found that the depletion of CD8 T cells or NK cells during the priming, but not the effector phase, led to reduced NK or CTL activities, respectively. These data suggest either indirect crosstalk between these cells mediated through DCs, or through direct NK cell/CD8 T cell interactions. Activated NK cells were reported to boost ongoing adaptive immune responses by producing IFN-γ which, in turn, promoted Th1 polarization,[29] and CD8 T cells are the major effector cells in Th1

responses. Furthermore, Ag-specific T cells can directly activate NK cells by secreting IL-2,[30] which also provides signals required by NK cells in "helping" DCs.[31]

In contrast to the ability of NK cells and CD8 T cells to positively regulate DCs as well as each other, CD4 T cells appear to negatively regulate these cells. In our experiments, CD4 depletion during the priming phase led to the activation of DCs, NK cells and CD8 T cells, as well as enhanced NK activity. These data suggest the existence of $CD4^+CD25^+$ Treg cells, which over the past several years have reached the spotlight in tumor immunology. Depletion of $CD4^+CD25^+$ T cells in mice were reported to improve tumor clearance[32] and enhance the response to immunotherapy.[33] Furthermore, tumor infiltrating Treg cells were found to be clinically associated with reduced survival of cancer patients.[34] In our previous experiments, we showed that depletion of CD4 T cells during the effector phase led to a better protection of mice against tumor challenge,[10] thereby suggesting not only the presence of Treg, but also that these cells are negatively regulating vaccine efficacy. $CD4^+CD25^+$ Treg cells, similar to all $CD4^+$ T cells, do not express the NKG2D receptor even after their activation.[4] This could indeed explain the lack of enhancement of Treg cell activation and activity observed in our experiments, despite the profound activation of DCs, NK, and CD8 T cells. Thus, by engaging the NKG2D receptor, our vaccine achieved preferential activation of NK and $CD8^+$ T cells, and thus may have tilted the balance towards immune surveillance and breakage of Treg-mediated peripheral tolerances to tumor Ags. However, it is also possible that some $CD4^+$ T cell subpopulations are required for optimal immune responses and the generation of effective immune response and memory as previously reported.[35,36] Specifically, the lack of these subpopulations may explain the reduction in CT-26 tumor cell killing after CD4

depletion during the priming phase. The finding that CD4 T cell depletions during the effector phase also reduced CTL activity suggests that CD4 T cell help is also required for the maintenance of Ag-specific CD8 T cells *in vivo* as previously suggested.[37] Alternatively, the reduction in CTL activity observed in our experiments may also be the result of a lack in CD4 T cell help in tissue culture prior to ^{51}Cr release assays.

Peyer's patches are believed to be the location for T cell priming induced by DNA vaccines delivered by attenuated *Salmonella typhimurium*.[16,17] The homing of lymphocytes, mediated by selectins, chemokine-receptors and integrins, is achieved by a complex, multi-step process, including capture and rolling, firm adhesion and subsequent emigration.[38] One could assume that compared to the pBud control vaccine, the pH60/Surv vaccine would stimulate CD8 T cells through TCRs in the presence of costimulatory signals provided by the NKG2D receptor. This may have been the primary reason for the robust activation of CD8 T cells observed in our experiments. In contrast, CD4 T cell activation is predicted to be largely non-specific and due to bystander effects from LPS or cytokines, and would thus explain our finding that this activation was only moderate. These differences in initial signals and extent of activation may have contributed to the different expression profiles observed in our experiments where CD4 T cells showed moderate upregulation of PSGL-1 and $\alpha 4\beta 7$ integrin, which were shown to be induced by cytokines and LPS. [39,40] In contrast, CD8 T cells displayed a much stronger upregulation of PSGL-1, CCR7 and αE integrin, which were reported to be upregulated by signaling through the TCR.[20,41,40,42]

L-selectin,[43] CCR7[44] and $\alpha 4\beta 7$ integrin[45] were reported to be important homing receptors involved in lymphocyte recruitment to Peyer's patches under normal

circumstances. The downregulation of L-selectin on lymphocytes observed in our experiments may have been the result of their activation, since activation leads to rapid down-regulation of surface L-selectin expression on lymphocytes.[18] The upregulation of CCR7, especially on CD8 T cells, might reflect their robust activation since CCR7 was reported to be transiently upregulated on T cells following mitogen or anti-CD3 Ab treatment.[41] Alternatively, it could also be the result of a greater percentage of naïve CD8 T cells homing to Peyer's patches, since CCR7 are highly expressed on naïve T cells and mediate these cells' homing to secondary lymphoid tissues such as the Peyer's patches.[44] If this is the case, these findings may reflect that the pH60/Surv vaccine, delivered by attenuated *S. typhimurium*, also induced changes in Peyer's patch stromal cells especially since these cells are the source of ligands of CCR7, i.e. chemokines CCL19 and CCL21.[44] We also observed that CD4 T cells displayed a slight upregulation of $\alpha4\beta7$. However, the reduced number of CD4 T cells in Peyer's patches, despite their increased expression of $\alpha4\beta7$ integrin, suggests that other homing receptors may also contribute significantly to the lymphocyte homing induced by the pH60/Surv DNA vaccine.

P-selectin is normally absent from Peyer's patches, but under certain conditions, can be expressed on HEV of Peyer's patches.[46] In our experiments, the 'inflammatory' conditions created in Peyer's patches could have induced the expression of P-selectin in the Peyer's patches, particularly since endotoxin was shown to induce P-selectin expression.[47] Thus the upregulation of functional PSGL-1, especially in the presence of IL-12 secreted by activated DCs, might contribute to the homing of different lymphocytes to Peyer's patches and Th1 polarization, as previously suggested.[39] Furthermore, upon binding their ligands, some homing receptors, such as CCR7[48],

αEβ7[49], PSGL-1[50], were reported to mediate co-stimulatory signal and induce Th1 polarization. In our experiments, the changes of homing receptor expression profiles, and presumably changes on stromal cells, including CCL19, CCL21 and P-selectin expression, could actually favor the retention of partially activated cells and the attraction of more Th1 type cells. Therefore such changes could lead to an increase in positive contributors and a decrease in negative regulators homing to Peyer's patches, and thus add another dimension to the crosstalk induced by the pH60/Surv DNA vaccine.

The lymphocytes that are activated in the Peyer's patches, due to their homing receptor expression, are likely to home to the periphery where they can combat tumor cells or become quiescent in the absence of Ag. Their exact fate is difficult to track down at this time due to the lack of specific and stable markers for these lymphocytes.

In summary, we demonstrated that by preferentially activating and attracting positive regulators and reducing negative regulators in Peyer's patches, the pH60/Surv DNA vaccine induced increased lymphocyte crosstalk, and thereby established a microenvironment more suitable for NK cell activation and T cell priming. The success of this vaccine in combating tumors of different origins and NKG2D expression levels in both prophylactic and therapeutic models,[10] and inducing a long-lived immune memory, shows that activation of both the innate and adaptive arms of the immune system represents an attractive strategy to overcome tumor-induced peripheral tolerance.

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Figure legends:

Figure 1. Activation of DCs, NK and T cells in Peyer's Patches following vaccination. Mice were sacrificed 1 d after vaccination. Lymphocytes isolated from Peyer's patches were analyzed by flow cytometry. (A) Contour plots of CD80 and CD86 expression on DCs ($CD11c^+$ I-A/I-E^+). (B) Contour plots of CD69 and NKG2D expression by NK cells ($CD3^-DX5^+$). (C) Contour plots of CD69 and NKG2D expression by $CD8^+$ T cells ($CD3^+$ $CD8^+$). Vectors used for vaccination are shown on top of (A). Experiments were repeated 3 times with similar results.

Figure 2. Depletion of CD8 T cells during priming results in reduction of DC activation and NK cell activity. (A) Lymphocytes were isolated from Peyer's patches according to Fig. 1 and analyzed on the $CD11c^+$ I-A/I-E^+ population. Shaded areas indicate pBud control mice, with their average mean fluorescence intensity (MFI) and standard deviation shown as the gray numbers, while black line and black number represent pH60/Surv-vaccinated mice. Left panels: no CD8 depletion, right panels: CD8 depletion. Numbers indicate average of MFI and standard deviation of two samples within the group in the same experiment. All overlay histograms are presented as % of Max on the Y-axis. *, $P<0.01$ compared to pBud control group. (B) Mice vaccinated with pH60/Surv were either undepleted (none, solid squares) or depleted of CD8 T cells starting on 1 d before the 1st vaccination (Pre-vac., shaded circles) or 3 d after the 2nd vaccination (Post-vac., open circles). Mice were sacrificed 2 wks after the 2nd vaccination. Freshly isolated splenocytes were used in a standard ^{51}Cr-release assay against Yac-1 NK target cells. Upper panels indicated the DX5 and CD4 distribution among splenocytes from either undepleted or CD8 depleted mice. *, $P<0.01$ compared to

non-depletion or post-vac depletion groups. (C) Splenocytes freshly isolated from vaccinated mice were either undepleted (solid squares) or depleted of NK cells (open diamonds) and used in a standard ^{51}Cr-release assay against Yac-1 cells. *, $P<0.01$ compared to non-depletion group. All experiments were repeated at least once with similar results.

Figure 3. Depletion of NK cells during priming results in reduced DC activation and CTL activity. Mice were treated similarly as in Fig. 2, except that NK cell depletion was performed as described in Material and Methods. (A) Histograms of CD80 and CD86 distribution of $CD11c^+$ $I\text{-}A/I\text{-}E^+$ cells isolated from Peyer's patches with (right panels) or without (left panels) NK cell depletion. *, $P<0.01$ compared to pBud control group. **, $P<0.05$ compared to pBud control group depleted of NK cells. (B) Mice vaccinated with pH60/Surv were either not depleted (solid squares) or depleted of NK cells pre-vaccination (shaded diamonds) or post-vaccination (open diamonds). Splenocytes were stimulated *in vitro* with irradiated CT-26 colon carcinoma cells for 5 d and used in a standard ^{51}Cr-release assay against these tumor cells. Upper panels indicate the distribution of CD4 and CD8 T cells obtained from either undepleted or NK cell depleted mice. *, $P<0.05$ compared to non-depletion or post-vac depletion groups. (C) *In* vitro stimulated splenocytes isolated from vaccinated mice either not depleted (solid squares) or depleted of CD8 cells (open circles) and used in ^{51}Cr release assay against CT-26 target cells. *, $P<0.01$ compared to non-depletion group. All experiments were repeated at least once with similar results.

Figure 4. Depletion of CD4 T cells enhances lymphocyte activation and NK cell activity. Mice were treated similarly as in Fig. 2, except that CD4 depletion was

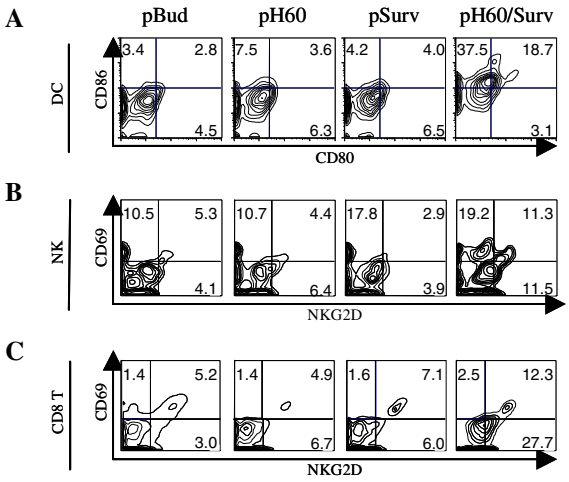
performed as described in Material and Methods. (A) Histograms of the activation markers of lymphocytes isolated from Peyer's patches of pH60/Surv-vaccinated mice either with (dark lines) or without (shaded areas) CD4 depletion. Left panel was gated on $CD11c^+$ I-A/I-E^+ cells; middle panel was gated on $CD3^+CD8^+$ cells; and the right panel was gated on $CD3^-DX5^+$ cells. *, $P<0.01$ compared to non-depletion group. All increases were confirmed in a separate experiment utilizing another 2 mice in each group. (B) and (C) Mice vaccinated with pH60/Surv were not depleted (solid squares) or depleted of CD4 T cells pre-vaccination (shaded triangles) or post-vaccination (open triangles). (B) Freshly isolated splenocytes were used in a standard ^{51}Cr-release assay against Yac-1 NK target cells. Upper panels indicate the CD8 and DX5 distribution of splenocytes obtained from either undepleted or CD4 depleted mice. *, $P<0.05$ compared to non-depletion or post-vac depletion groups. (C) Splenocytes were stimulated *in vitro* for 5 d with irradiated CT-26 cells and used in a standard ^{51}Cr-release assay against these tumor cells. For both pre-vac and post-vac depletion groups: *, $P<0.05$ compared to non-depletion group. All experiments were repeated at least once with similar results.

Figure 5. The pH60/Surv vaccine induces changes in expression of lymphocyte homing receptors and lymphocyte distribution in Peyer's patches. Lymphocytes were isolated from Peyer's patches and analyzed by flow cytometry. (A) Histograms depict homing receptor expression of lymphocytes isolated from Peyer's patches of pBud control (shaded area) or pH60/Surv-vaccinated (black line) mice. Upper panels are gated on $CD3^+CD4^+$ cells, and the lower panels on $CD3^+CD8^+$ cells. Experiment was repeated once with similar results. (B) Percentage of different lymphocyte populations in Peyer's Patches. Shaded bars: pBud control mice; black bars: pH60/Surv-vaccinated mice.

*P<0.05 compared to the pBud control group of mice. Experiment was repeated 3 times with similar results.

Figure 6. The pH60/Surv vaccine reduces the percentage of $CD4^+CD25^+$ T cells in Peyer's patches without their activation. (A) Percentage of $CD4^+CD25^+$ cells in Peyer's patches. *P<0.05 compared to the pBud control group. (B) Expression of CD25 (left panel), CD69 (middle panel) and GITR (right panel) on $CD3^+CD4^+CD25^+$ cells. Shaded area: pBud control group; black line: pH60/Surv group. Experiment was repeated once with similar results. (C) Inhibition of CD3-induced splenocyte proliferation by $CD4^+CD25^+$ cells. CFSE-labeled splenocytes were cultured with anti-CD3 Ab in the absence (shaded area) or presence of unlabeled $CD3^+CD4^+CD25^+$ cells (purity are over 99% as determined by flow cytometry) at a ratio of 100:1. The $CD3^+CD4^+CD25^+$ cells were isolated either from pBud control mice (black dotted line) or pH60/Surv vaccinated mice (gray solid line).

Figure 7. The pH60/Surv vaccine induces long-term protection against tumor cell challenge. Mice were challenged by i.v. injection of $1x10^5$ CT-26 colon carcinoma cells 5 months after the 2nd vaccination and sacrificed 20 d later. (A) Average lung weight of challenged mice. *P<0.02 compared to pBud control group. The average lung weight of normal mice is approximately 0.2g. (B) Splenocytes were stimulated *in vitro* for 5 d with irradiated CT-26 cells and then used in a standard ^{51}Cr-release assay against CT-26 target cells. *P<0.01 compared to pBud control group.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pBud
pH60
pSurv
pH60/Surv
DC
CD86
CD80
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4.5
7.5
3.6
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4.2
4.0
6.5
37.5
18.7
3.1
B
NK
CD69
NKG2D
10.5
5.3
4.1
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4.4
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17.8
2.9
3.9
19.2
11.3
11.5
C
CD8 T
CD69
NKG2D
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7.1
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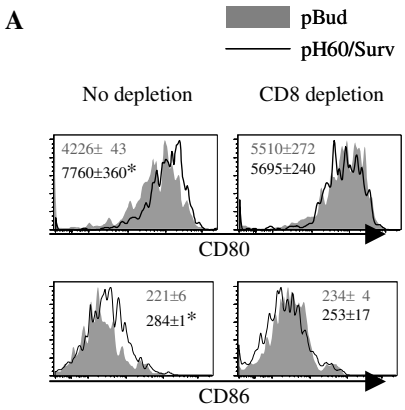
2.5
12.3
27.7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pBud
pH60/Surv
No depletion
CD8 depletion
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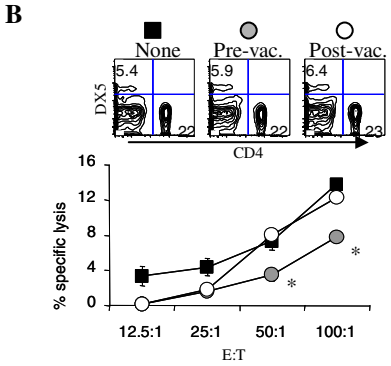
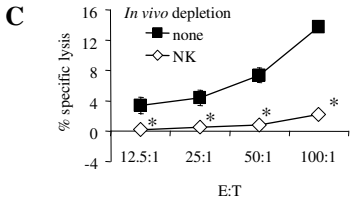
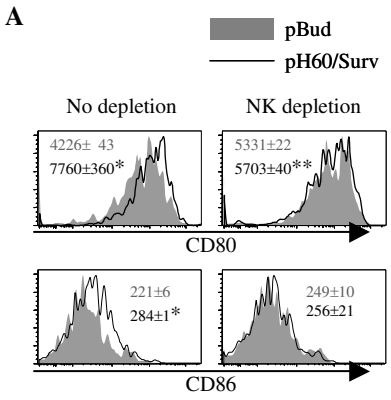
7760±360*
5510±272
5695±240
CD80
221±6
284±1*
234± 4
253±17
CD86


B
None
Pre-vac.
Post-vac.
5.4
5.9
6.4
DX5
CD4
% specific lysis
E:T
12.5:1
25:1
50:1
100:1


C
In vivo depletion
none
NK
% specific lysis
E:T
12.5:1
25:1
50:1
100:1


A
pBud
pH60/Surv
No depletion
NK depletion
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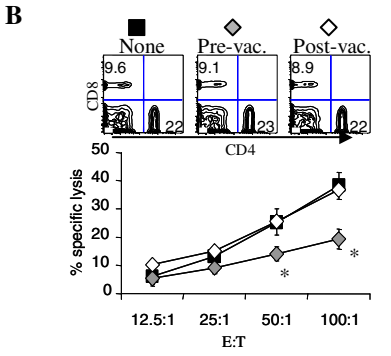
7760±360*
5331±22
5703±40**
CD80
221±6
284±1*
249±10
256±21
CD86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None
Pre-vac.
Post-vac.
9.6
9.1
8.9
CD8
22
23
22
CD4
% specific lysis
50
40
30
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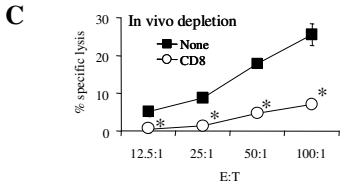
10
0
12.5:1
25:1
50:1
100:1
E:T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In vivo depletion
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CD8
% specific lysis
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20
10
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12.5:1
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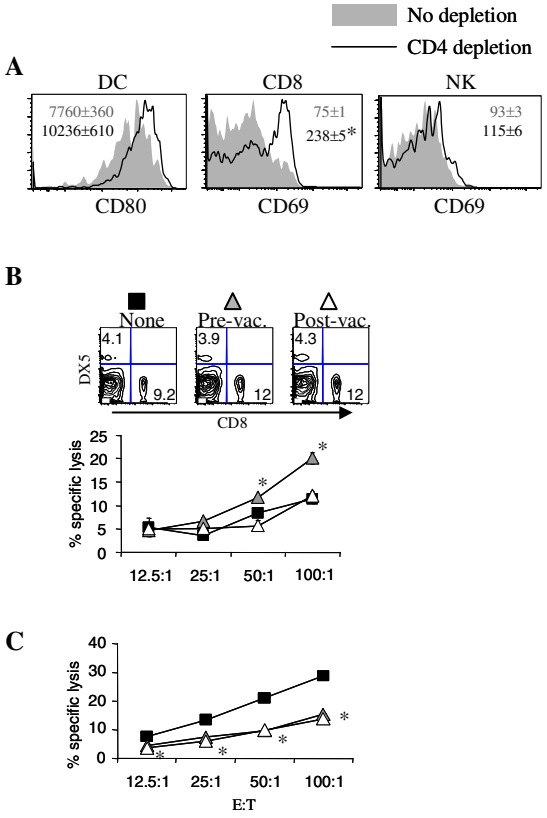
50:1
100:1
E:T


No depletion
CD4 depletion
A
DC
CD8
NK
7760±360
10236±610
75±1
238±5*
93±3
115±6
CD80
CD69
CD69
B
None
Pre-vac.
Post-vac.
4.1
3.9
4.3
9.2
12
12
DX5
CD8
% specific lysis
25
20
15
10
5
0
12.5:1
25:1
50:1
100:1
C
% specific lysis
40
30
20
10
0
12.5:1
25:1
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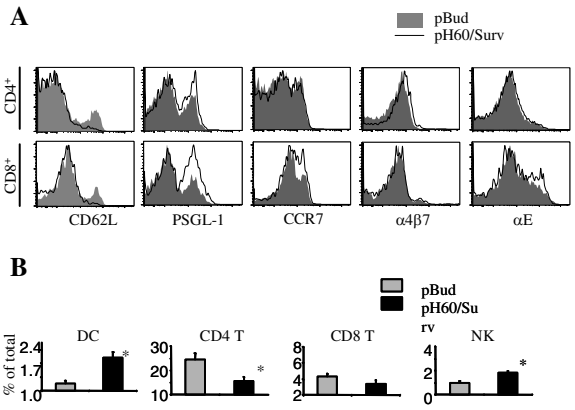
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pBud
pH60/Surv
CD4+
CD8+
CD62L
PSGL-1
CCR7
α4β7
αE
B
pBud
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% of total
DC
CD4 T
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NK
2.4
1.7
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30
20
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8
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0
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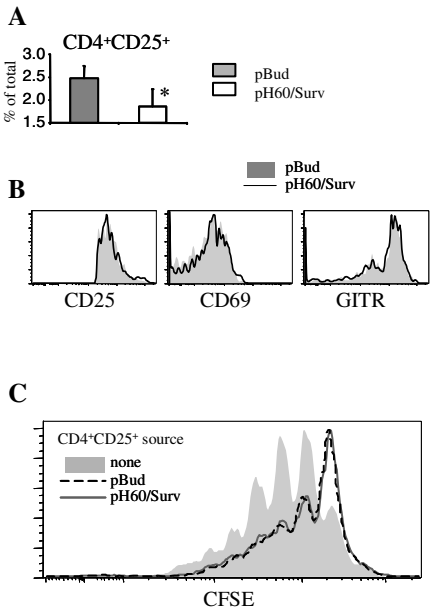
A
CD4+CD25+
3.0
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pBud
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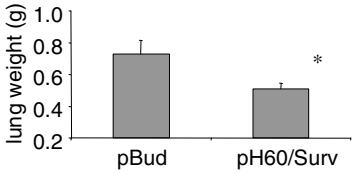
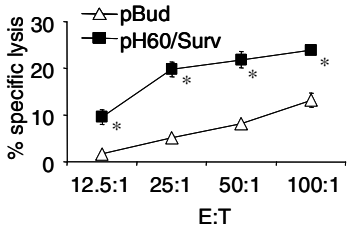
none
pBud
pH60/Surv
CFSE

A


lung weight (g)
1.0
0.8
0.6
0.4
0.2
pBud
pH60/Surv
*

B


pBud
pH60/Surv
% specific lysis
30
20
10
0
12.5:1
25:1
50:1
100:1
E:T
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Review

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DNA Vaccines Designed to Inhibit Tumor Growth by Suppression of Angiogenesis

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Key Words

DNA vaccines · Antiangiogenesis · Cell-mediated immunity · Tumor metastases · Receptor tyrosine kinase · Transcription factor

Abstract

The development of new blood vessels, i.e. angiogenesis, is a rate-limiting step in the development of tumors since tumor growth is generally limited to 1–2 mm^3 in the absence of a blood supply. Thus, the inhibition of tumor growth by attacking the tumor's vascular supply offers a primary target for antiangiogenic intervention by DNA-based vaccines. Here, we describe two novel orally delivered DNA vaccines which suppress tumor angiogenesis and induce a robust cell-mediated immune response that provides for long-lived protection against melanoma, colon, breast and non-small-cell lung carcinoma in mouse model systems. These vaccines, which are delivered by attenuated *Salmonella typhimurium* to secondary lymphoid organs, are directed against such targets as vascular endothelial growth factor receptor 2 (FLK-1) and transcription factor Fos-related antigen 1 (Fra-1). Both vaccines break peripheral T cell tolerance against these self-antigens and induce a robust T cell-mediated immune response leading to suppression of tumor angiogenesis and resulting in effective suppression of tumor growth and metastases. Such research efforts may open up new possibilities for the rational design of future DNA vaccines effective for the prevention and treatment of cancer.

Introduction

The development and universal use of vaccines against infectious disease have been highly successful because they are capable of inducing long-lived antibody responses which protect against highly immunogenic bacteria and viruses. However, vaccinations against intracellular organisms such as agents of tuberculosis, malaria and HIV require cell-mediated immunity and are either not available or not uniformly effective [1]. Vaccines against cancer face an even more difficult challenge since their targets are usually tumor-associated self-antigens that are poorly immunogenic and frequently heterogeneously expressed by genetically unstable tumor cells that undergo mutations and often suppress immune responses at the cellular level. Consequently, a new form of vaccination

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that uses DNA containing the gene encoding the target antigen of the vaccine has been developed during the last decade and is now under intensive investigation. A number of excellent and comprehensive reviews on such DNA vaccines have been published recently that cover various aspects of this extensive area of research [2–8]. Consequently, this paper does not provide yet another such review, but focuses instead entirely on novel DNA-based cancer vaccines developed and evaluated in the authors' laboratory during the last few years. Particular emphasis is placed on a strategy which features oral vaccine delivery with attenuated *Salmonella typhimurium* to secondary lymphoid organs, polyubiquitination for optimal antigen processing, coexpression of cytokines and targeting of both tumor cells and their vasculature. A major challenge for DNA vaccines against cancer is to break peripheral T cell tolerance against tumor self-antigens and to induce a robust, long-lived T cell-mediated protective tumor immunity which leads to an effective suppression of tumor angiogenesis. These DNA vaccines were designed to be effective both in prophylactic settings, in which tumor cell growth and metastases induced by a subsequent tumor cell challenge are suppressed, and in therapeutic settings, where already established metastases are eradicated in animal tumor models. Here, we describe and evaluate two different DNA-based oral cancer vaccines which induce T cell-mediated immune responses leading to antiangiogenesis and suppression of tumor growth and metastasis. The first is a DNA vaccine specifically directed against the tumor vasculature of 3 different solid tumors by targeting vascular endothelial growth factor (VEGF) receptor 2 (FLK-1), which is overexpressed by proliferating endothelial cells but not by tumor cells, and the second is a DNA vaccine which coexpresses IL-18 and is directed against the transcription factor Fos-related antigen 1 (Fra-1), which is overexpressed in breast tumor cells.

A DNA Vaccine against FLK-1 Designed to Inhibit Tumor Growth by Suppression of Angiogenesis

Rationale

The inhibition of tumor growth and metastasis by attacking the tumor's vascular supply offers a primary target for antiangiogenic intervention. This approach, pioneered by Folkman and colleagues [9–13], is attractive for several reasons. First, since the inhibition of tumor-associated angiogenesis is a physiological host mechanism, it is unlikely to lead to the development of resistance. Second, because each tumor capillary can potentially supply hundreds of tumor cells, targeting the tumor vasculature can substantially potentiate antitumor effects. Third, direct contact of the vasculature with the circulation leads to efficient access of therapeutic agents. In this regard, studies by several investigators have already established a central role for angiogenesis in the invasion, growth and metastasis of solid tumors [10, 14–16]. In fact, angiogenesis is a rate-limiting step in the development of tumors, since tumor growth is generally limited to 1–2 mm^3 in the absence of a blood supply [17, 18]. Beyond this minimum size, tumors often become necrotic and apoptotic under such circumstances.

The receptor tyrosine kinases and their growth factor ligands are required for tumor growth and thus offer a relevant approach to suppress tumor angiogenesis. The VEGF receptor 2, also known as FLK-1, is among these receptors which bind the 5 isomers of murine VEGF and has a more restricted expression on endothelial cells. In fact, FLK-1 is upregulated once these cells proliferate during angiogenesis in the tumor vasculature. Thus, this growth factor receptor is strongly implicated as a relevant therapeutic target, especially since it is required for tumor angiogenesis and plays an important role in tumor growth, invasion and metastasis [14, 15, 18–20]. In this regard, several strategies have been applied to block FLK-1, including dominant-negative receptor mutants, germline disruption of VEGF receptor genes, monoclonal antibodies against VEGF and a series of synthetic receptor tyrosine kinase inhibitors [21, 22]. We decided to develop a novel strategy for achieving an antitumor immune response with an FLK-1-based DNA vaccine which causes the collapse of tumor vessels by evoking a T cell-mediated immune response against proliferating endothelial cells, leading to the suppression of tumor angiogenesis and eradication of tumor growth and metastasis.

Expression Plasmids for FLK-1 and Protein Expression

The FLK-1 expression vector was constructed by inserting the DNA encoding the entire murine FLK-1 gene into the pcDNA3.1 vector between the restriction sites *Kpn*I (5′) and *Xba*l (3′). This construct was verified by nucleotide sequencing, and protein expression was established by Western blotting after transient transfection into COS-7 cells. The protein appeared in the lysate of these cells in its glycosylated form at 220 kD and to a lesser extent in its unglycosylated form at approximately 150 kD [23].

Selection of DNA Vaccine Delivery Vehicle

The use of attenuated strains of *S. typhimurium* as a DNA vaccine carrier is based on initial findings by Hoiseth and Stocker [24]. A timely, recent review by Dietrich et al. [25] details the extensive use of a number of live attenuated bacteria as vectors to deliver plasmid DNA vaccines [25].

Work with such attenuated (AroA$^-$) bacteria indicated that they could be used for oral somatic transgene vaccination [26] and to trigger the elicitation of antigen-specific humoral, T helper and cytotoxic responses against β-galactosidase, a model antigen [27]. Importantly, since professional antigen-presenting cells (APCs) play a key role in the induction of effective immune responses evoked by vaccination with plasmid DNA, the use of attenuated intracellular bacteria as a delivery vehicle has the potential to efficiently target DNA vaccines to professional APCs.

We initially found the attenuated (AroA$^-$) strain SL 7207 of *S. typhimurium*, made available by B.A.D. Stocker (Stanford University), to be an effective carrier for oral delivery by gavage of an autologous DNA vaccine which effectively protected against a lethal challenge with murine melanoma cells. In fact, this vaccine broke peripheral T cell tolerance toward murine melanoma self-antigens gp100 and TRP-2 containing the murine ubiquitin gene fused to minigenes encoding peptide epitopes $gp100_{25–33}$ and TRP-$2_{181–188}$ and induced a robust, tumor-specific CD8+ T cell response, resulting in suppression of melanoma tumor growth [28]. Following gavage, these live, attenuated bacteria transport the DNA through the small intestine and then through the M cells that cover the Peyer's patches of the gut. From there, the attenuated bacteria enter APCs such as dendritic cells (DCs) and macrophages, which are plentiful in this secondary lymphoid organ, where they die because of their AroA$^-$ mutation, liberating multiple copies of the DNA inside these phagocytes. There, the DNA is transcribed and translated to protein which is then processed in the proteasomes of these APCs. This is followed by the formation of peptide-MHC class I antigen complexes, which are ultimately presented by the APCs to the T cell receptor of naïve T cells. This entire process is depicted schematically in figure 1. Thus far, we have successfully used attenuated *S. typhimurium* as a carrier for several of our DNA vaccines encoding the human carcinoembryonic antigen gene, effective in eliciting potent CD8+ T cell immunity in carcinoembryonic antigen-transgenic mice that eradicated growth and metastases of colon [29] and non-small-cell lung carcinomas [30]. This same approach was also used successfully to deliver plasmids encoding genes for VEGF receptor 2 (FLK-1) to Peyer's patches, resulting in a robust T cell-mediated immune response against proliferating endothelial cells in the tumor vasculature in 3 different tumor models. This led ultimately to effective suppression of angiogenesis and subsequent eradication of tumor metastases in both prophylactic and therapeutic settings [23].

Our rationale for delivering DNA vaccines to Peyer's patches orally by gavage with attenuated *S. typhimurium* was strongly supported by results of a recent study by Maloy et al. [31], clearly indicating that intralymphatic immunization is the most effective means to strongly enhance DNA vaccination. This was evident from a comparison of conventional routes of immunization, i.e. intradermal, intramuscular or intrasplenic, with intralymphatic immunizations of DNA encoding cytotoxic T lymphocyte epitopes of the highly immunogenic lymphocytic choriomeningitis virus glycoprotein. In this case, direct injection of the DNA into a peripheral lymph node enhanced immunogenicity by 100- to 1,000-fold, inducing strong and biologically relevant CD8+ cytotoxic T lymphocyte responses.

The successful transfer of expression vectors encoding FLK-1 to Peyer's patches in the small intestine was demonstrated when mice were administered by gavage 10^8 colony-forming units (CFU) of attenuated *S. typhimurium* transfected with enhanced green fluorescent protein (EGFP), and 24 h thereafter were killed and biopsies were collected from the thoroughly washed intestine. As shown in figure 2, these bacteria harboring EGFP exhibited strong fluorescence in the Peyer's patches. Thus, these attenuated bacteria can transfer the target gene to Peyer's patches, and the plasmids which encode each individual gene can successfully express their respective protein. The attenuated bacteria do not survive very long because neither EGFP activity nor the bacteria were detected in immunized mice after 72 h.

The FLK-1 Vaccine Suppresses Tumor Growth and Metastasis

The hypothesis that an FLK-1-based DNA vaccine can inhibit tumor growth was validated by demonstrating that this could be readily achieved in murine tumor models of melanoma, colon and non-small-cell lung carcinoma. For example, marked inhibition of subcutaneous tumor growth was observed in mice challenged 2 weeks after the 3rd oral vaccination with pcDNA3.1-FLK-1, carried by attenuated *S. typhimurium,* by a subcutaneous challenge with murine melanoma cells or non-small-cell lung carci-

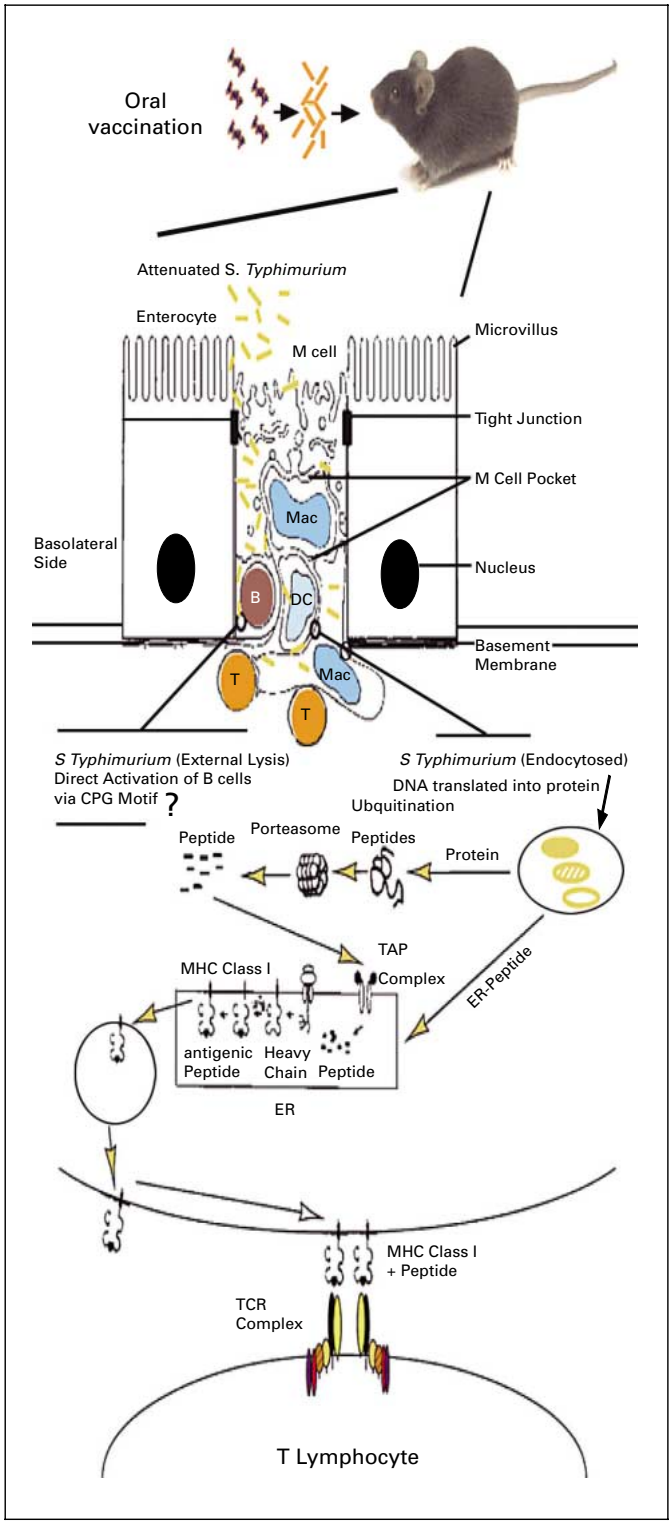

Fig. 1. Schematic diagram of mechanisms of action elicited by DNA vaccines delivered by attenuated *S. typhimurium* to Peyer's patches. T = T cells; B = B cells; Mac = macrophages; DC = dendritic cells.

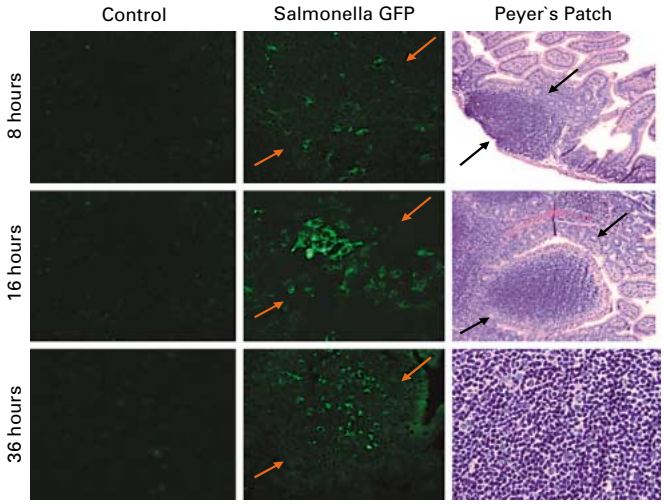

Fig. 2. Direct evidence for DNA transfer from attenuated *S. typhimurium* to Peyer's patches. Mice were immunized by gavage with 10^8 CFU of attenuated *S. typhimurium.* Fluorescence expression of EGFP was detected by confocal microscopy and is depicted in the middle column. Hematoxylin-eosin staining of mouse Peyer's patches is shown in the right column.

noma cells. In contrast, animals injected with only the empty vector carried by the attenuated bacteria revealed uniformly rapid tumor growth. Most importantly, prolonged antitumor effects were demonstrated in the MC-38 murine colon carcinoma model as long as 10 months after their last vaccination, as all animals showed essentially no tumor growth compared with controls when subjected to a tumor cell challenge at this time point [23].

Suppression of spontaneous and established metastases could also be achieved since protection against spontaneous pulmonary metastases of non-small-cell lung carcinoma was quite evident 30 days after surgical excision of subcutaneous primary tumors by either the absence of metastases or their marked suppression. Importantly, vaccination prolonged the life span of these mice 4-fold. In addition, possible resistance against the vaccine was ruled out by rechallenging survivors 120 days after their 1st tumor cell challenge, with 4 of 5 mice not revealing any tumor [23]. Remarkably, the FLK-1 vaccine was also able to reduce dissemination of established spontaneous pulmonary metastases of CT-26 colon carcinoma cells in a therapeutic setting. In this case, when mice were vaccinated 10 days after the establishment of experimental metastases, all such animals survived and showed only very few small lung foci, whereas all control mice treated with the empty vector or with PBS began to die 28 days after tumor cell challenge [23].

Immunological Mechanisms Induced by the FLK-1 Vaccine

Mechanism studies indicated clearly that CD8+ T cells were primarily responsible for the antitumor response achieved by the FLK-1 vaccine. This was indicated by a marked increase in T cell activation markers of splenocytes from successfully vaccinated mice after a 12-hour incubation with B16G3.26 melanoma cells that had been stably transduced to express FLK-1. These findings included increased expression of CD25, the high-affinity IL-2 receptor α chain, CD69, an early T cell activation antigen, and LFA-2 (CD2), a lymphocyte function-associated antigen. This upregulation of activation markers was clearly evident when compared with CD8+ T cells from mice vaccinated with pcDNA3.1-FLK1 but incubated with wild-type B16G3.26 melanoma cells. Specific recognition of FLK-1 was evident, as no increase in the expression of activation markers was noted following coincubation of cells expressing FLK-1 with splenocytes from C57BL/6J mice vaccinated with the empty vector. No such upregulation could be observed for CD4+ T cells [23]. The involvement of CD8+ T cells in the antitumor immune response has been clearly demonstrated, since in vivo depletion of such cells prior to intravenous challenge of vaccinated mice with CT-26 tumor cells resulted in the complete abrogation or severe impairment of the antitumor response. In fact, mice depleted of CD8+ T cells died within 45 days after tumor cell challenge due to extensive growth and dissemination of pulmonary metastases. However, in vivo depletion of CD4+ T cells did not decrease the effectiveness of the vaccine [23].

In addition, vaccination against FLK-1 did induce a robust T cell-mediated lysis that was demonstrated by antigen-specific cytotoxicity against CT-26 colon carcinoma cells, transduced with FLK-1, as observed in a 4-hour ^{51}Cr release assay. Importantly, specificity was indicated, as wild-type CT-26 cells not expressing FLK-1 were not lysed [23]. Recent experiments demonstrated that murine endothelial cells expressing FLK-1 were also specifically lysed by CD8+ T cells from mice successfully vaccinated with the FLK-1 vaccine. Furthermore, the tumor endothelium, marked by endothelial cells visualized with rhodamine-conjugated anti-CD31 antibody, was demonstrated to be the target of the FLK-1 vaccine, as FITC-labeled CD8+ T cells from vaccinated mice were clearly evident in these tissues. In contrast, almost no CD8+ T cells were observed in nonvascularized, viable areas of tumor tissues, even 4 months after tumor cell challenge, nor were they associated with vessels in somatic tissue [23].

Suppression of Angiogenesis Induced by the FLK-1 Vaccine

The marked reduction in neovascularization following successful treatment with the FLK-1 vaccine clearly demonstrated its distinct antiangiogenic effect. This was shown to be independent of tumor cells in a Matrigel assay where such differences were visible macroscopically in Matrigel plugs that were removed from mice 6 days after their implantation. In this case, vascularization was induced either by VEGF or basic fibroblast growth factor, and vessel growth was quantified after in vivo staining of endothelium with FITC-labeled Isolectin B4 and subsequent evaluation of extracts by fluorimetry [23]. Reduction in neovascularization was also clearly evident from the extent of vascularization evaluated by relative fluorescence after in vivo staining of endothelium with FITC-conjugated lectin. There was a marked decrease in VEGF or basic fibroblast growth factor-induced neovascularization only after vaccination with the vector encoding FLK-1 but not with the empty vector. Immunohistochemical staining with anti-CD31 antibody further revealed a decrease in vessel density among pulmonary metastases of CT-26 colon carcinoma after successful vaccination when compared with tissue derived from control mice [23].

Since angiogenesis is required for wound healing and is important for fertility, additional experiments were required to assess any possible damaging effects of the FLK-1 vaccine. A measurable prolongation was noticed in the time required to completely close a total of 24 circular wounds inflicted on the backs of 6 mice immunized with the FLK-1-based vaccine compared to that in mice immunized with the control vector (14.75 days, SD 1.5 days, versus 13.3 days, SD 1.6 days; $p < 0.01$). This was accompanied by macroscopically visible swelling and inflammation in 11 of the 24 FLK-1 vaccine-immunized mice versus 4 of 24 cases among controls [23].

However, the wounds of mice that were subjected to tumor excisions, including in some cases opening of the peritoneum, healed without any complications. Additional experiments revealed no impact on fertility of mice based on the time elapsed from start of cohabitation until parturition or on the number of pups born. All females of each experimental group gave birth. Neuromuscular performance as determined by both the wire test and footprint test, as well as by body weight, overall behavior and balancing tests, did not demonstrate any impairment attributable to vaccination. Finally, the occurrence of common, FLK-1-positive progenitor cells for both endothelial cells and hematopoietic cells necessitated an evalu-

ation of peripheral blood samples of C57BL/6J and BALB/c mice up to 10 months after their last immunization with the FLK-1 vaccine. In this case, total blood counts and differentials did not indicate any decreased or compensating hematopoiesis [23].

A DNA Vaccine Encoding Transcription Factor Fra-1 and Secretory IL-18

Rationale

The rationale for selecting the transcription factor Fra-1 as a DNA vaccine target was based on several considerations. First, a limited number of transcription factors are generally overactive in most cancer cells, which makes them appropriate targets for anticancer drugs, provided selective inhibition of transcription can be applied rather than general inhibition, which is expected to be too toxic [32]. In fact, rather than selecting specific inhibitors of a transcription factor, Fra-1 was chosen since it belongs to the transcription factor activating protein-1 family, which defines tumor progression and regulates breast cancer cell invasion and growth as well as resistance to antiestrogens. In addition, Fra-1 is overexpressed by many human and mouse epithelial carcinoma cells, including breast cancer cells [33]. This overexpression of Fra-1 greatly influences these cells' morphology and motility, correlates with their transformation to a more invasive phenotype and is specifically associated with highly invasive breast cancer cells. These findings suggest Fra-1 to be a potentially useful target for active immunization against breast cancer [34].

The rationale for coexpressing IL-18 with a Fra-1 vaccine is also based on several considerations. First, IL-18 is a potent immunoregulatory cytokine and an IFN-γ-inducing factor that enhances cytokine production of T and/or natural killer (NK) cells and induces their proliferation and cytolytic activity [34]. Tumor cells engineered to produce IL-18 resulted in considerable therapeutic activity in several mouse models [34]. Furthermore, IL-18 enhances cellular immune mechanisms by upregulating MHC class I antigen expression and favoring the differentiation of CD4+ helper T cells toward the T helper 1 subtype [35]. In turn, T helper 1 cells secrete IL-2 and IFN-γ, which facilitate the proliferation and/or activation of CD8+ cytotoxic T lymphocytes, NK cells and macrophages, all of which can contribute to tumor regression [36]. In addition, IL-18 is a novel inhibitor of angiogenesis, sufficiently potent to suppress tumor growth by directly inhibiting fibroblast growth factor 2-induced endothelial cell proliferation [37]. The role of recombinant IL-18 as a biological 'adjuvant' has been recently evaluated in murine tumor models, and its systemic administration induced significant antitumor effects in several tumor models [37, 38]. Finally, there is a rationale for the development of prophylactic Fra-1-IL-18 cancer vaccines since considerable data from experimental systems have shown that immunity can be activated to prevent tumors. Thus, there is a strong rationale for prevention, since in such a setting, one deals with an immune system which is unimpaired by immune suppression induced by tumors and/or treatment. Neither is there tolerance to tumor antigens that were confronted in the absence of appropriate antigen presentation and costimulatory signals. In such a setting, the use of overexpressed growth factor receptors or transcription factor-related antigens yields rational targets for specific immune prevention, also in individuals whose tumors were eradicated by standard therapies. The rationale for developing such a prophylactic vaccine has thus guided the research efforts described here for the Fra-1-IL-18 vaccine.

Construction and Protein Expression of the pFra-1-pIL-18 Vaccine

Two constructs were made based on the pIRES vector. The first, pUb-Fra-1, was comprised of polyubiquitinated, full-length murine Fra-1. The second, pIL-18, contained murine IL-18 with an Ig kappa leader sequence for secretion purposes. The empty vector with or without ubiquitin served as a control [34]. As pointed out above, polyubiquitination has been used for all our DNA vaccines, particularly since we found in one of our initial studies that the presence of ubiquitin upstream of a DNA minigene encoding melanoma peptide epitopes proved to be essential for achieving tumor-protective immunity [39]. Also, based on a vast body of literature on the role of ubiquitin in protein processing in the proteasome [40], this molecule was considered to be essential for optimizing antigen processing and ultimately effective antigen presentation.

Protein expression of pUb-Fra-1 and pIL-18 was demonstrated by transient transfection of each vector into COS-7 cells and by performing Western blots of the respective cell lysates (pUb-Fra-1 or IL-18) and supernatant (pIL-18) with anti-Fra-1 and anti-IL-18 antibody, respectively. All constructs produced protein of the expected molecular mass, with IL-18 being expressed in its active form at 18 kD and Fra-1 as a 46-kD protein. Protein expression of IL-18 was also detected in the culture supernatant of transfected cells. Importantly, the biofunc-

tional activity of IL-18 was clearly demonstrated by ELISA in supernatants of cells transfected with pIL-18 [34].

Furthermore, the attenuated *S. typhimurium* successfully transferred expression vectors to mouse Peyer's patches. Thus, DNA encoding pUb-Fra-1 and pIL-18 was effectively released from the attenuated bacteria and entered Peyer's patches in the small intestine. The DNA was subsequently transcribed by APCs, processed in the proteasome and presented as MHC-peptide complexes to T cells, as illustrated in figure. 1. To this end, mice were administered by gavage 1×10^8 CFU of dam$^-$, AroA$^-$ attenuated *S. typhimurium*. After 24 h, these animals were killed and biopsies were collected from the thoroughly washed small intestine. The doubly attenuated bacteria harboring EGFP exhibited strong EGFP fluorescence [34]. This finding suggests not only that such bacteria can transfer the target gene to Peyer's patches, but also that plasmids encoding each individual gene can successfully express their respective proteins. Importantly, these doubly attenuated bacteria do not survive very long because neither EGFP activity nor live bacteria could be detected in immunized animals after 72 h. However, EGFP expression was detected in adherent cells, most likely APCs, such as DCs and macrophages from Peyer's patches after oral administration of *S. typhimurium* harboring the eukaryotic EGFP expression plasmid. Taken together, these findings indicate that both plasmid transfer to and protein expression in eukaryotic cells did take place [34].

Induction of Tumor-Specific Protective Immunity

The hypothesis was tested that an orally administered DNA vaccine encoding murine pUb-Fra-1 together with secretory pIL-18 (pUb-Fra-1-pIL-18), carried by attenuated *S. typhimurium,* can induce protective immunity against subcutaneous tumors and experimental pulmonary metastases of D2F2 breast carcinoma. In fact, a marked suppression of disseminated pulmonary metastases was observed in BALB/c mice challenged by an intravenous injection of D2F2 tumor cells, 1 week after the last of 3 vaccinations at 2-week intervals with pUb-Fra-1-pIL-18. A marked increase in tumor volume of subcutaneously injected D2F2 cells also resulted when this vaccine was applied and compared to a number of controls. Importantly, the life span of 60% of successfully vaccinated BALB/c mice (5/8) was tripled in the absence of any detectable tumor growth up to 11 weeks after tumor cell challenge [34]. In addition, breast cancer cells were killed in vitro by both tumor-specific cytotoxic T lymphocytes and NK cells. Thus, CD8+ T cells isolated from splenocytes of mice immunized with the vaccine encoding pUb-Fra-1-pIL-18 effectively killed D2F2 breast cancer cells in vitro in a ^{51}Cr release assay. In contrast, such T cells isolated from control animals were ineffective. Thus, cytotoxic T lymphocyte-mediated killing was specific since syngeneic prostate cancer target cells (RM-2) were not lysed. Furthermore, the CD8+ T cell-mediated tumor cell lysis was MHC class I antigen-restricted, since addition of anti-H2K^d/H-2D^d antibody abrogated tumor cell lysis. NK cells were also effective in killing D2F2 tumor cells in an assay against Yac-1 target cells, in contrast to control immunizations, which were ineffective [34].

Immunological Mechanisms Induced by the Vaccine

Interactions between IL-18 and active T helper 1 and NK cells were found to be critical for achieving both optimal antigen-specific T cell and NK cell responses. Thus, the pUb-Fra-1-pIL-18 vaccine or pIL-18 alone upregulated the expression of the respective T and NK cell markers. This was evident from fluorescence-activated cell sorting analysis (FACS) analyses indicating a marked increase (10.4%) over the empty vector control in expression of CD25, the high-affinity IL-2 receptor α chain, CD69 on early T cell activation antigen (14.1%), CD28 (12.2%) and CD11a (17.1%), all of which are important for initial interactions between T cells and DCs, as well as regular T cell markers CD4+ (14.5%) and CD8+ (16.1%). Since it is known that NK cells can also play a role in antitumor immune responses, we tested spleen cells from immunized and control mice with anti-DX5 antibody and found DX5 expression, important for NK cytotoxicity, to be markedly increased from 2 to 35.3% [34].

This increase found in costimulatory molecules expressed by DCs was significant, since it is well known that T cell activation depends on upregulated expression of the costimulatory molecules CD80 and CD86 on DCs to achieve optimal ligation with CD28 on activated T cells. In fact, FACS of splenocytes from successfully immunized mice and controls indicated that expression of CD80 and CD86 was markedly upregulated on CD11c+ DCs by the vaccine by 10 and 9.5%, respectively [34]. The activation of T cells by the pFra-1-pIL-18 vaccine was further demonstrated by their increased secretion of IFN-γ and IL-2. It is a well-known fact that the release of these two proinflammatory cytokines from T cells indicates their activation in secondary lymphoid tissues. An analysis for these cytokines, both intracellularly with flow cytometry or at the single cell level by ELISPOT, indi-

cated that vaccination with the pUb-Fra-1-pIL-18 plasmid and a subsequent challenge with D2F2 tumor cells resulted in marked increases of IFN-γ and IL-2 release over that induced by control vaccines [34].

Suppression of Angiogenesis

Antiangiogenic effects were evaluated 2 weeks after the last oral vaccination with the pUb-Fra-1-pIL-18 DNA plasmid in mice, injected subcutaneously in the sternal region with growth factor-reduced Matrigel containing murine fibroblast growth factor 2 and irradiated D2F2 tumor cells. Six days later, the endothelium of these animals was stained by intravenous injection of fluorescent *Bandeiraea simplicifolica* lectin I, and 30 min thereafter, Matrigel plugs were excised for macroscopic evaluation. Lectin-FITC was then extracted from these plugs and quantified by fluorimetry. Indeed, distinct suppression of angiogenesis induced by the DNA vaccine was demonstrated in this Matrigel assay by a marked decrease in vascularization. This was evident from evaluation of fluorescence after in vivo staining of the endothelium of mice with FITC-conjugated lectin. In fact, such differences were visible macroscopically in representative Matrigel plugs removed from vaccinated mice 6 days after their injection. Suppression of angiogenesis was clearly evident from FITC-lectin staining, indicating decreased vascularization in Matrigel plugs after vaccination with the pUb-Fra-1-pIL-18 vaccine and to a lesser extent with pIL-18, but not with control vaccines [34].

Conclusions and Future Perspectives

Both DNA-based cancer vaccines described here induced a T cell-mediated immune response sufficiently robust to break peripheral T cell tolerance against such self-antigens as FLK-1 and transcription factor Fra-1, which resulted in the effective generation of tumor-protective immunity. This immunological effector mechanism appears to be dominant, irrespective of the vaccine target antigen. A common feature of both DNA vaccines is the suppression of angiogenesis induced by the T cell-mediated destruction of proliferating endothelial cells in the tumor vasculature overexpressing these vaccine target antigens. A common denominator for the two DNA-based vaccines is their oral delivery vehicle of attenuated *S. typhimurium,* which targets them to secondary lymphoid organs considered to be optimal for induction of antitumor immune responses induced by DNA vaccines [41]. Furthermore, coexpression of polyubiquitin, which targets proteins to the proteasome, was effective for optimal antigen processing in all of these DNA vaccine applications. Robust suppression of angiogenesis in the tumor vasculature proved especially effective with both the DNA vaccine targeting FLK-1 overexpressed on proliferating endothelial cells [23], and to some extent also for the Fra-1-based vaccine, particularly due to its coexpression of IL-18 [34]. The Fra-1-IL-18-based DNA vaccine was primarily effective in inducing tumor protection in a prophylactic setting, whereas the FLK-1 vaccine was also able to markedly suppress both spontaneous and established tumor metastases in a therapeutic setting. The long-lived (up to 10 months) protective immunity induced by the FLK-1 vaccine was particularly impressive. The vaccine, which targets FLK-1, has several advantages by specifically eliciting a CD8+ T cell-mediated immune response against proliferating endothelial cells in the tumor vasculature rather than directly against tumor cells. First, endothelial cells are genetically stable and do not downregulate MHC class I and II antigens, an event that frequently occurs in solid tumors and severely impairs T cell-mediated antitumor responses [42]. In addition, immune suppression triggered by tumor cells at the cellular level can also be avoided by this approach. Second, the therapeutic target is tumor independent; thus, killing of proliferating endothelial cells in the tumor microenvironment can be effective against a variety of malignancies. Furthermore, proliferating endothelial cells are readily available to lymphocytes in the bloodstream. Consequently, the target tissue can be reached unimpaired by anatomical barriers such as the blood-brain barrier or encapsulation of tumor tissues [43].

Future perspectives should also include DNA-based cancer vaccines which not only suppress tumor angiogenesis but also jointly induce effective tumor cell apoptosis. One such candidate DNA vaccine is currently under investigation in the authors' laboratory and targets the inhibition of apoptosis protein survivin, which is overexpressed in both tumor cells and proliferating endothelial cells in the tumor vasculature [44]. Another important target for DNA-based cancer vaccines is the tumor stroma, where the fibroblast-activating protein, overexpressed on fibroblasts in the tumor stroma, represents a particularly attractive target. However, in order to prevent tumor recurrence or effectively treat established human cancer, it is most likely necessary to follow up such novel DNA vaccines with chronically administered chemotherapy at metronomic, i.e. lower than maximum tolerated dose, dose levels [45]. Such a combination therapy might be of considerable interest, particularly since Bocci

et al. [46] reported recently that thrombospondin 1, a mediator of antiangiogenic effects, is induced by prolonged in vitro exposure of endothelial cells to metronomic doses of chemotherapy. Such combined approaches could ultimately lead to the rational design of novel and effective modalities for the treatment of cancer.

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DNA vaccines suppress tumor growth and metastases by the induction of anti-angiogenesis

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Summary: Four novel oral DNA vaccines provide long-lived protection against melanoma, colon, breast, and non-small cell lung carcinoma in mouse model systems. The vaccines are delivered by attenuated *Salmonella typhimurium* to secondary lymphoid organs and are directed against targets such as carcinoembryonic antigen, tyrosine-related protein, vascular endothelial growth factor receptor-2 [also called fetal liver kinase-1 (FLK-1)], and transcription factor Fos-related antigen-1 (Fra-1). The FLK-1 and Fra-1 vaccines are effective in suppressing angiogenesis in the tumor vasculature. All four vaccines are capable of inducing potent cell-mediated protective immunity, breaking peripheral T-cell tolerance against these self-antigens resulting in effective suppression of tumor growth and metastasis. It is anticipated that such research efforts will contribute toward the rational design of future DNA vaccines that will be effective for prevention and treatment of human cancer.

Introduction

The development and universal use of vaccines against infectious disease have been among the great accomplishments of biomedical science. The primary reason for the success of these vaccines is that they are capable of inducing long-lived antibody responses that protect against highly immunogenic bacteria and viruses. However, vaccination against intracellular organisms that require cell-mediated immunity, such as agents of tuberculosis, malaria, and human immunodeficiency virus (HIV), are either not available or not uniformly effective (1). Vaccines against cancer face an even greater challenge, because their targets usually consist of tumor-associated self-antigens which are poorly immunogenic and often heterogeneously expressed by genetically unstable tumor cells that undergo mutations and frequently suppress immune responses at the cellular level. For these reasons, a new form of vaccination, using DNA that contains the gene encoding for the target antigen of interest, has been developed during the last decade and is now under intense investigation. This article does not provide a review of this extensive area of research but focuses

instead entirely on novel DNA-based cancer vaccines developed and evaluated in the authors' laboratory during the last few years. Particular emphasis is placed on a novel strategy that features oral vaccine delivery with attenuated *Salmonella typhimurium*, polyubiquitination for optimal antigen processing, co-expression of costimulatory molecules or cytokines, and targeting of both tumor cells and their vasculature. The major challenge for DNA vaccines against cancer is to overcome peripheral T-cell tolerance against tumor self-antigens and to induce a robust, long-lived, T-cell-mediated protective tumor immunity, including an effective suppression of tumor angiogenesis. Although the majority of our DNA vaccines were effective in a prophylactic setting suppressing metastases induced by subsequent tumor cell challenges, a few vaccines were also capable of suppressing the dissemination of already established experimental tumor metastases in therapeutic animal models.

Here, we will describe three different approaches for DNA-based oral cancer vaccines that induce cell-mediated immune responses resulting in suppression of tumor growth and metastasis. These will include the following: (i) DNA vaccines against human carcinoembryonic antigen (CEA) that exclusively target colon and non-small cell lung carcinoma cells overexpressing this oncofetal antigen; (ii) a DNA vaccine specifically directed against the tumor vasculature of four different solid tumors which suppress tumor angiogenesis by targeting vascular endothelial growth factor receptor-2 (VEGFR-2), also known as fetal liver kinase-1 (FLK-1), which is overexpressed by proliferating endothelial cells but not by tumor cells; and (iii) a DNA vaccine which co-expresses interleukin (IL)-18 and is directed against the transcription factor Fos-related antigen-1 (Fra-1) overexpressed in breast tumor cells, which induces T- and natural killer (NK)-cell-mediated anti-tumor immunity as well as suppression of tumor angiogenesis.

Carcinoembryonic antigen-based DNA vaccines

Rationale

We initially selected CEA as a vaccine target, because this well-characterized human oncofetal antigen is overexpressed on colon, breast, and non-small cell lung carcinoma cells but only poorly detectable on most normal adult tissues. In addition, CEA, a glycoprotein membrane antigen of 180–200 kDa (2, 3), had already been used to construct a DNA vaccine for immunotherapy by strategies different from ours (4, 6). Moreover, we had a useful model available for evaluating CEA-based vaccines that was provided by the establishment of a mouse line that carries the genomic CEA transgene for human CEA (7, 8). This CEA transgenic mouse model expresses CEA in a tissue-specific manner, similarly to humans, in which the colon is the main site of CEA production. Importantly, anti-CEA $CD8^+$ T cells could be elicited in these CEA transgenic mice after *in vivo* priming with CEA-transfected fibroblasts (9). Because anti-CEA antibody responses were not elicited unless an independent carrier was used, there is an apparent tolerance to CEA in the $CD4^+$ T-cell compartment of these mice (9).

Expression plasmids for CEA and protein expression of CEA and CD40 ligand trimer

Distinct forms of expression plasmids included those targeting CD40 ligand trimer (CD40LT) and CEA molecules to dendritic cells (DCs) or T cells, respectively. The pER-CEA control plasmid targeted to and remained in the endoplasmic reticulum (ER), whereas the pW-CEA plasmid targeted to the cell surface, as confirmed by confocal microscopy (10). Western blotting of lysates from COS-7 cells transfected with either of the two expression vectors indicated that both express CEA protein at the correct molecular size of 180 kDa (10). The plasmid encoding the *CD40LT* gene (pCD40LT) contained a modified 33-amino acid leucine zipper motif to facilitate the formation of trimeric CD40L that was fused to the C-terminus of the IL-7 sequence to direct protein expression to the cell surface. Western blotting indicated the plasmid pCD40LT-CEA to contain the entire CEA extracellular domain fused to the C-terminus of murine CD40, thus generating this dual function chimeric construct (11).

S. typhimurium transfers expression vectors to mouse Peyer's patches

These experiments are based on the working hypothesis that orally administered attenuated *S. typhimurium*, carrying expression vectors encoding CEA, pass from the stomach to the small intestine and through M cells into host Peyer's patches. This secondary lymphoid organ harbors immature DCs, B cells, T cells, NK cells, and macrophages, i.e. most of the important effector cells necessary for an immune response induced by a DNA vaccine. The attenuated bacteria are taken up by DCs and macrophages, and these antigen-presenting cells (APCs) are activated by the pathogen and start to differentiate, migrating to inductive sites of the immune system such as lymph nodes and spleen. During this time, the attenuated bacteria die due to their inability to synthesize aromatic amino acids, releasing a large number of plasmids that are subsequently transferred

into the cytosol and nucleus of the infected cells. Eventually, the encoded genes will be transcribed, translated, and processed through the proteasome of these host APCs and presented as major histocompatibility complex (MHC) class I–peptide complexes to T-cell receptors (TCRs) of naïve T cells (10, 11). This entire process is diagrammatically represented by *Fig.* 1. The transfer of expression vectors to Peyer's patches in the small intestine was demonstrated when mice were administered by gavage 10^8 CFU-attenuated *S. typhimurium* transfected with enhanced green fluorescence protein (EGFP), and 24 h thereafter the mice were killed and biopsies collected from the thoroughly washed intestine. As shown in *Fig.* 2, these bacteria harboring EGFP exhibited strong fluorescence in the Peyer's patch. Thus, these attenuated bacteria can transfer the target gene to Peyer's patches, and the plasmids which encode each individual gene can successfully express their respective protein. The attenuated bacteria do not survive very long, because neither EGFP activity nor the bacteria were detected in immunized mice after 72 h (10, 11).

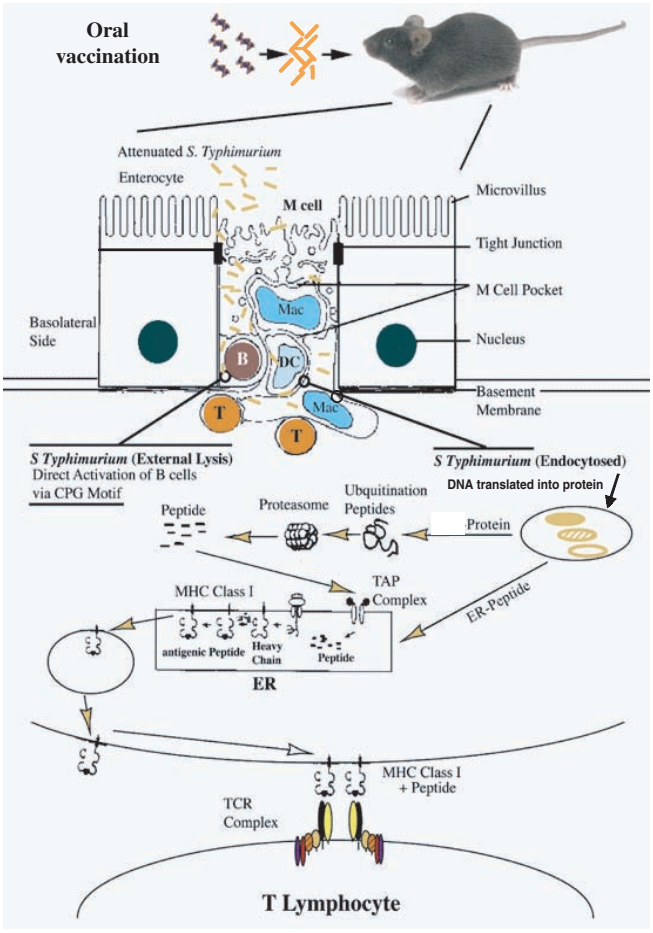

***Fig.* 1. Schematic diagram of mechanisms of action elicited by DNA vaccines delivered by attenuated *Salmonella typhimurium* to Peyer's patches.**

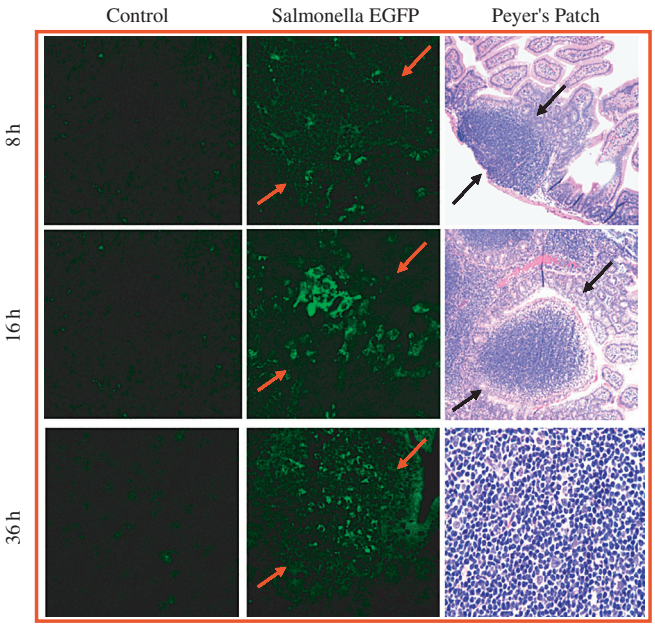

***Fig.* 2. Direct evidence for DNA transfer from attenuated *Salmonella typhimurium* to Peyer's patches.** Mice were immunized by gavage with 10^8 CFU of attenuated *S. typhimurium*. Fluorescence expression of EGFP was detected by confocal microscopy and is depicted on the left. Hematoxylin/eosin staining of mouse Peyer's patches is shown on the right.

Induction of tumor-protective immunity by CEA-based DNA vaccines

Because antigens are expressed in the cytosol, a cellular compartment for MHC class I presentation, there should be a strong induction of cytotoxic $CD8^+$ T cells. This response should induce a cell-mediated immune response in CEA-transgenic mice and break peripheral T-cell tolerance against a lethal challenge of MC38 murine colon carcinoma cells, stably transfected with CEA. This was indeed the case when such mice were immunized with three oral administrations at 2-week intervals with the pW-CEA vaccine and received a lethal subcutaneous challenge of 2.5×10^5 MC38-CEA colon carcinoma cells 2 weeks after the last vaccination. Four of eight mice completely rejected the tumor cell challenge 4 weeks thereafter, while the remaining animals revealed a fourfold reduction in tumor growth when compared to controls, such as mice receiving either irradiated MC38-CEA cells, empty vector, or pER-CEA designed to be maintained in the ER, all of which showed rapid and uniform tumor growth in all animals (10). The tumor-protective efficacy of this pW-CEA vaccine could be markedly increased, when the mice received five intravenous boosts of the recombinant anti-epithelial cell adhesion molecule (EpCAM) antibody–IL-2 fusion protein (5 μg each) on five consecutive days beginning 1 day after tumor cell challenge. This immunocytokine boost resulted in six of eight mice completely rejecting the tumor cell challenge,

with the two remaining animals showing a 10-fold reduction in tumor growth compared to controls (10).

In another application, an oral DNA vaccine against CEA prevented growth as well as dissemination of Lewis lung carcinoma metastases in CEA-transgenic mice. However, in this case, the vaccine boosted with the recombinant anti-EpCAM antibody–IL-2 fusion protein, as described above for colon carcinoma, induced an MHC class I antigen-restricted $CD8^+$ T-cell-mediated immune response that protected 100% of experimental CEA-transgenic mice from a lethal challenge of non-small cell lung carcinoma cells (12). In fact, none of the vaccinated mice produced subcutaneous tumors, while all control animals revealed massive tumors of $>2000\,mm^3$ 30 days after tumor cell challenge. Importantly, this tumor model, which responded better to the vaccine than the colon carcinoma model, also revealed the complete prevention of experimental pulmonary metastases in 75% of successfully vaccinated CEA-transgenic mice (12).

A dual function DNA vaccine encoding CEA and CD40LT proved to be most effective in inducing T-cell-mediated tumor-protective immunity in the CEA-transgenic mouse model. In fact, this vaccine, when further boosted by the anti-EpCAM–IL-2 fusion protein, achieved the complete rejection of a lethal colon carcinoma cell challenge in 100% of experimental animals. This dual function vaccine effectively activated both DCs and naïve T lymphocytes (11).

Mechanisms of immune responses induced by CEA-based DNA vaccines

The immunological response induced by the CEA-based DNA vaccines, particularly when boosted by the recombinant antibody–IL-2 fusion protein, involved primarily the action of highly activated MHC class I antigen-restricted $CD8^+$ T cells. Activation of these cytotoxic T lymphocytes (CTLs) was indicated by their markedly increased secretion of the proinflammatory cytokines interferon-γ (IFN-γ) and IL-12 and by robust upregulation of the T-cell activation markers CD25, CD28, CD69, and leukocyte function-associated antigen-1 (LFA-1). The marked increase of CD28 on T cells and of B7.1 and B7.2 costimulatory molecules on DCs is particularly important, because the activation of naïve T cells requires two independent signals. Firstly, binding of the peptide–MHC complex by the TCR produces a signal to T cells that indicates antigen recognition. Secondly, ligation of CD28 on activated T cells with B7.1 or B7.2 on DCs produces a second signal, which initiates T-cell responses and production of armed T-effector cells. Indeed, the marked elevation in the production of cytokines IFN-γ and IL-12 by T cells induced by our dual function DNA vaccine encoding CEA and CD40LT suggests that a third signal may act directly on T cells (13–15). This danger signal was reported to be required for T helper 1 (Th1) differentiation leading to a clonal expansion of T cells (15). In fact, whenever T-cell help is required to generate an effective $CD8^+$ T-cell response against a tumor self-antigen like CEA, triggering of DCs is necessary before their encounter with an antigen-specific $CD8^+$ T cell (16). This effect is mediated by ligation of CD40 on the surface of APCs (17) with CD40LT, which is expressed on activated $CD4^+$ T cells. Thus, CD40LT expressed by our DNA vaccine likely acted as a surrogate for activated $CD4^+$ T cells, leading to maturation of DCs, as indicated by their upregulation of B7.1 and B7.2 costimulatory molecules (18). Taken together, our orally administered dual function CEA vaccine encoding for both CEA and CD40LT induced a highly efficient tumor-protective immunity against the CEA self-antigen in 100% of experimental CEA-transgenic mice, particularly when additionally boosted by the anti-EpCAM antibody–IL-2 fusion protein (11).

DNA minigene vaccines

Among melanoma-associated antigens recognized by T cells are gp100, tyrosinase-related protein-1 (TRP-1), and TRP-2, which are lineage-specific differentiation antigens expressed by both melanocytes and melanoma cells (19, 20). In an attempt to determine the requirements for breaking peripheral T-cell tolerance to such naturally expressed self-antigens, attenuated *S. typhimurium* was used as a carrier for oral delivery of eukaryotic expression vectors encoding the ubiquitinated $gp100_{25-33}$ and TRP-$2_{181-188}$ peptide epitopes. Following three immunizations with this DNA vaccine at 2-week intervals and a challenge 1 week thereafter by subcutaneous injection with 1×10^5 B16G3.26 murine melanoma cells, B57BL/6J mice showed effective tumor-protective immunity as tumor growth was at least fourfold to fivefold suppressed when compared to empty vector controls (19). Importantly, ubiquitination was found absolutely essential to achieve tumor-protective immunity, because immunization with the autologous minigene lacking the coding sequence for murine ubiquitin resulted in large tumor growth in all mice equal to that of controls (19, 20).

The mechanism of action of this minigene vaccine was demonstrated by adoptive transfer of $CD8^+$ T cells from successfully immunized mice to syngeneic severe combined immunodeficiency disease (scid) mice. After subsequent

challenge of these mice with wildtype B16G3.26 melanoma cells, all vaccinated animals showed a >60% reduction in tumor growth compared to control animals, which received $CD8^+$ T cells from mice vaccinated with only the empty ubiquitin vector (19). In addition, only $CD8^+$ T cells from successfully vaccinated mice induced MHC class I antigen-restricted CTL-mediated killing of melanoma cells *in vitro*. Activation of these T cells by the vaccine was further indicated by an eightfold increase in their IFN-γ release over that of control lymphocytes (19, 20).

The data obtained with this melanoma minigene vaccine further supported the contention that DNA immunization can be enhanced significantly by exploiting the natural pathways of antigen presentation. Thus, most peptides presented by MHC class I antigens that induce CTLs are derived from cytosolic proteins degraded by the proteasome, a process in which many copies of the cellular protein ubiquitin are covalently attached to the target protein. The rationale for encoding polyubiquitin in DNA vaccines is based on the findings obtained with the melanoma minigene vaccine as well as data by several investigators indicating that cotranslational expression of modified ubiquitin with lymphocytic choriomeningitis virus nucleoprotein resulted in more rapid degradation of this nucleoprotein by the proteasome and a much improved anti-viral immunity than did a vaccine lacking ubiquitin (21, 22).

DNA vaccines designed to inhibit tumor growth by suppression of angiogenesis

Rationale

The inhibition of tumor growth and metastasis by attacking the tumor's vascular supply offers a primary target for anti-angiogenic intervention. This approach, pioneered by Folkman and colleagues (23–27), is attractive for several reasons. Firstly, the inhibition of tumor-associated angiogenesis is a physiological host mechanism and should not lead to the development of resistance. Secondly, each tumor capillary has the potential to supply hundreds of tumor cells, so that targeting the tumor vasculature actually potentiates the anti-tumor effect. Thirdly, direct contact of the vasculature with the circulation leads to efficient access of therapeutic agents. In fact, studies by many investigators established that angiogenesis has a central role in the invasion, growth, and metastasis of solid tumors (24, 28–30). Thus, angiogenesis is a rate-limiting step in the development of tumors, because tumor growth is generally limited to 1–2 mm^3 in the absence of a blood supply (31, 32). Beyond this minimum size, tumors often become necrotic and apoptotic under such circumstances.

A molecularly defined approach to suppress tumor angiogenesis is offered by receptor tyrosine kinases (RTKs) and their growth factor ligands required for tumor growth. Among these receptors, the VEGFR-2, also known as FLK-1, binds the five isomers of murine VEGF and has a more restricted expression on endothelial cells. This growth factor receptor is upregulated once these cells proliferate during angiogenesis in the tumor vasculature. Thus, FLK-1 is strongly implicated as a therapeutic target, as it is necessary for tumor angiogenesis and has an important role in tumor growth, invasion, and metastasis (28, 29, 33–35). In fact, several approaches have been used to block FLK-1, including dominant-negative receptor mutants, germ-line disruption of *VEGFR* genes, monoclonal antibodies against VEGF, and a series of synthetic RTK inhibitors (35, 36). We developed a novel strategy for achieving an anti-tumor immune response with an FLK-1-based DNA vaccine. This vaccine causes the collapse of tumor vessels by evoking a T-cell-mediated immune response against proliferating endothelial cells overexpressing this growth factor receptor in the tumor vasculature.

Suppression of tumor growth and metastases

The hypothesis that an FLK-1-based DNA vaccine can inhibit tumor growth was validated by demonstrating that this could be readily achieved in murine tumor models of melanoma, colon, and non-small cell lung carcinoma. Marked inhibition of subcutaneous tumor growth was observed in mice challenged 2 weeks after the third oral vaccination with pcDNA3.1-FLK-1, carried by attenuated *S. typhimurium*, by subcutaneous challenge with murine melanoma cells or non-small cell lung carcinoma cells. In contrast, animals injected with only the empty vector carried by the attenuated bacteria revealed uniformly rapid tumor growth. Most importantly, prolonged anti-tumor effects were demonstrated in the MC-38 murine colon carcinoma model 10 months after their last vaccination, as all animals showed essentially no tumor growth compared with controls when subjected to a tumor cell challenge at this time point (37).

Suppression of spontaneous and established metastases

Protection against spontaneous pulmonary metastases of non-small cell lung carcinoma was also evident 30 days after surgical excision of subcutaneous primary tumors by either the absence of metastases or their marked suppression. Importantly, vaccination prolonged the lifespan of mice fourfold.

Possible resistance against the vaccine was ruled out by rechallenging survivors 120 days after their first tumor cell challenge, with four of five mice not revealing any tumor (37). Remarkably, the FLK-1 vaccine was also able to reduce established spontaneous pulmonary metastases of CT-26 colon carcinoma cells in a therapeutic setting. In this case, mice were vaccinated 10 days after the establishment of experimental metastases. All such treated mice survived and showed only very few small lung foci, whereas all control animals treated with the empty vector of phosphate-buffered saline began to die 28 days after tumor cell challenge (37).

Immunological mechanisms of the FLK-1 vaccine

Mechanism studies indicated clearly that $CD8^+$ T cells were primarily responsible for the anti-tumor response achieved by the FLK-1 vaccine. Thus, there was a marked increase in T-cell activation markers in splenocytes from successfully vaccinated mice after a 12-h incubation with B16G3.26 melanoma cells that had been stably transduced to express FLK-1. This finding included increased expression of CD25, the high-affinity IL-2 receptor α-chain, CD69, an early T-cell activation antigen, and LFA-2 (CD2). This upregulation was clearly evident when compared with $CD8^+$ T cells from mice vaccinated with pcDNA3.1-FLK-1 but incubated with wildtype B16G3.26 melanoma cells. Specific recognition of FLK-1 was indicated, as no increase in expression was noted following co-incubation of cells expressing FLK-1 with splenocytes from C57BL/6J mice vaccinated with the empty vector. No such upregulation could be observed for $CD4^+$ T cells (37). The involvement of $CD8^+$ cells in the anti-tumor immune response was demonstrated, as *in vivo* depletion of $CD8^+$ T cells before intravenous challenge of vaccinated mice with CT-26 tumor cells resulted in the complete abrogation or severe impairment of the anti-tumor response. Mice depleted of $CD8^+$ T cells died within 45 days after tumor cell challenge due to extensive growth and dissemination of pulmonary metastases. However, *in vivo* depletion of $CD4^+$ T cells did not decrease the effectiveness of the vaccine (37).

Vaccination against FLK-1-induced T-cell-mediated lysis, as demonstrated by antigen-specific cytotoxicity against CT26 colon carcinoma cells transduced with FLK-1, as observed in a 4-h ^{51}Cr-release assay. Wildtype CT26 cells not expressing FLK-1 were not lysed (37). Recent experiments demonstrated that murine endothelial cells expressing FLK-1 were also specifically lysed by $CD8^+$ T cells from mice successfully vaccinated with the FLK-1 vaccine. Furthermore, the tumor endothelium, marked by endothelial cells visualized with rhodamine-conjugated anti-CD31 antibody, was demonstrated to be the target of the FLK-1 vaccine, as FITC-labeled $CD8^+$ T cells from vaccinated mice were clearly evident in these tissues. In contrast, almost no $CD8^+$ T cells were observed in non-vascularized, viable areas of tumor tissues, even 4 months after tumor cell challenge, nor were they associated with vessels in somatic tissue (37).

Anti-angiogenesis induced by the FLK-1 vaccine

The marked reduction in neovascularization, following successful treatment with the FLK-1 vaccine, clearly demonstrated its distinct anti-angiogenic effect. This was shown to be independent of tumor cells in a Matrigel assay where such differences were visible macroscopically in Matrigel plugs removed 6 days after their installment. In this case, vascularization was induced either by VEGF or basic fibroblast growth factor (bFGF), and vessel growth was quantified after *in vivo* staining of endothelium with FITC-labeled isolectin B4 and evaluation by fluorimetry (37). Reduction in neovascularization was also evident from the extent of vascularization evaluated by relative fluorescence after *in vivo* staining of endothelium with FITC-conjugated lectin. There was a marked decrease in VEGF or bFGF-induced neovascularization, after vaccination with the vector encoding FLK-1 but not with the empty vector. Immunohistochemical staining with anti-CD31 antibody further revealed a decrease in vessel density among pulmonary metastases of CT-26 colon carcinoma after successful vaccination, when compared with tissue derived from control mice (37).

Because angiogenesis is required for wound healing and is important for fertility, experiments were done to assess possible damaging effect of the FLK-1 vaccine. A measurable prolongation was noticed in the time required to completely close a total of 24 circular wounds inflicted on the backs of six mice immunized with the FLK-1-based vaccine versus that of mice immunized with the control vector (14.75 days, SD 1.5, versus 13.3 days, SD 1.6; $P < 0.01$). Macroscopically visible swelling and inflammation was evident in 11 of 24 cases in treated mice versus four of 24 cases among controls (37). However, the wounds of mice that were subjected to tumor excisions, including in some cases opening of the peritoneum, healed without any complications. Additional experiments revealed no impact on fertility of mice based on the time elapsed from start of cohabitation until parturition or on the number of pups born. All females of each experimental group gave birth. Neuromuscular performance, as determined by both the wire test and footprint test, as well as by body weight, overall behavior, and balancing tests did not demonstrate any impairment attributed to vaccination. Finally, the occurrence

of common, FLK-1-positive progenitor cells for both endothelial cells and hematopoietic cells necessitated an evaluation of peripheral blood samples of C57BL/6J and BALB/*c* mice up to 10 months after their last immunization with the FLK-1 vaccine. In this case, total blood counts and differentials did not indicate any decreased or compensating hematopoiesis (37).

A DNA vaccine encoding transcription factor Fos-related antigen-1 and secretory IL-18

Rationale

The rationale for selecting the transcription factor Fra-1 as a DNA vaccine target was based on several considerations. Firstly, a limited number of transcription factors are generally overactive in most cancer cells, which makes them appropriate targets for anti-cancer drugs, provided selective inhibition of transcription can be applied rather than general inhibition, which is expected to be too toxic (38). In fact, rather than selecting specific inhibitors of a transcription factor, Fra-1 was chosen because it belongs to the transcription factor activating protein-1 (AP-1) family, which defines tumor progression and regulates breast cancer cell invasion and growth as well as resistance to anti-estrogens. In addition, Fra-1 is overexpressed by many human and mouse epithelial carcinoma cells, including breast cancer cells (39). This overexpression of Fra-1 greatly influences these cells' morphology and motility, correlates with their transformation to a more invasive phenotype, and is specifically associated with highly invasive breast cancer cells. These findings suggest that Fra-1 is a potentially useful target for active immunization against breast cancer (40).

The rationale for co-expressing IL-18 with a Fra-1 vaccine is also based on several considerations. Firstly, IL-18 is a potent immunoregulatory cytokine and an IFN-γ-inducing factor that enhances cytokine production of T and/or NK cells and induces their proliferation and cytolytic activity (40). Tumor cells engineered to produce IL-18 resulted in considerable therapeutic activity in several mouse models (40). Furthermore, IL-18 enhances cellular immune mechanisms by upregulating MHC class I antigen expression and favoring the differentiation of $CD4^+$ helper T cells toward the Th1 subtype (41). In turn, Th1 cells secrete IL-2 and IFN-γ, which facilitate the proliferation and/or activation of $CD8^+$ CTLs, NK cells, and macrophages, all of which can contribute to tumor regression (42). In addition, IL-18 is a novel inhibitor of angiogenesis, sufficiently potent to suppress tumor growth by directly inhibiting FGF2-induced endothelial cell proliferation (43). The role of recombinant IL-18 as a biological 'adjuvant' has been recently evaluated in murine tumor models, and its systemic administration induced significant anti-tumor effects in several tumor models (43, 44). Finally, there is a rationale for the development of prophylactic Fra-1/IL-18 cancer vaccines, because considerable data from experimental systems have shown that immunity can be activated to prevent tumors. Thus, there is a strong rationale for prevention, because in such a setting one deals with an immune system that is unimpaired by immune suppression induced by tumors and/or treatment. Neither is there tolerance to tumor antigens that were confronted in the absence of appropriate antigen presentation and costimulatory signals. In such a setting, the use of overexpressed growth factor receptors or transcription factor-related antigens yields rational targets for specific immune prevention, also in individuals whose tumors were eradicated by standard therapies. The rationale for developing such a prophylactic vaccine has thus guided the research efforts described here for the Fra-1/IL-18 vaccine.

Construction of the pFra-1/pIL-18 vaccine

Two constructs were made based on the pIRES vector. The first, pUb-Fra-1, was comprised of polyubiquitinated, full-length murine Fra-1. The second, pIL-18, contained murine IL-18 with an immunoglobulin κ (Igκ) leader sequence for secretion purposes. The empty vector with or without ubiquitin served as a control (40). As pointed out previously in this article, polyubiquitination has been used for all our DNA vaccines, particularly because we found in one of our initial studies that the presence of ubiquitin upstream of a DNA minigene encoding melanoma peptide epitopes proved to be essential for achieving tumor-protective immunity (19). Also, based on a vast body of literature on the role of ubiquitin in protein processing in the proteasome (45), this molecule was considered to be essential for optimizing antigen processing and ultimately effective antigen presentation.

Protein expression of pUb-Fra-1 and pIL-18 was demonstrated by transient transfection of each vector into COS-7 cells and by performing Western blots of the respective cell lysates (pUb-Fra-1 or IL-18) and supernatant (pIL-18) with anti-Fra-1 and anti-IL-18 Ab, respectively. All constructs produced protein of the expected molecular mass, with IL-18 being expressed in its active form at 18 kDa and Fra-1 as a 46 kDa protein. Protein expression of IL-18 was also detected in the culture supernatant of transfected cells. Importantly, the biofunctional activity of IL-18 was clearly demonstrated by enzyme-linked immunosorbent assay in supernatants of cells transfected with pIL-18 (40).

The attenuated *S. typhimurium* successfully transferred expression vectors to mouse Peyer's patches. Thus, DNA encoding

pUb-Fra-1 and pIL-18 was effectively released from the attenuated bacteria and entered Peyer's patches in the small intestine. The DNA was subsequently transcribed by APCs, processed in the proteasome, and presented as MHC–peptide complexes to T cells, as illustrated by *Fig. 1*. To this end, mice were administered 1×10^8 CFU of dam$^-$AroA$^-$-attenuated *S. typhimurium* by gavage. After 24 h, these animals were killed and biopsies collected from the thoroughly washed small intestine. The doubly attenuated bacteria harboring EGFP exhibited strong EGFP fluorescence (40). This finding suggests not only that such bacteria can transfer the target gene to Peyer's patches, but also that plasmids encoding each individual gene can successfully express their respective proteins. Importantly, these doubly attenuated bacteria do not survive very long, because neither EGFP activity nor live bacteria could be detected in immunized animals after 72 h. However, EGFP expression was detected in adherent cells, most likely APCs such as DCs and macrophages from Peyer's patches after oral administration of *S. typhimurium* harboring the eukaryotic EGFP expression plasmid. Taken together, these findings indicate that both plasmid transfer to and protein expression in eukaryotic cells did take place (40).

Induction of tumor-specific protective immunity

The hypothesis was tested that an orally administered DNA vaccine encoding murine pUb-Fra-1 together with secretory pIL-18 (pUb-Fra-1/pIL-18), carried by attenuated *S. typhimurium*, can induce protective immunity against subcutaneous tumors and experimental pulmonary metastases of D2F2 breast carcinoma. A marked suppression of disseminated pulmonary metastases was observed in BALB/*c* mice, challenged 1 week after the third vaccination at 2-week intervals with pUb-Fra-1/pIL-18, by an intravenous injection of D2F2 tumor cells. A marked increase in tumor volume of subcutaneous D2F2 also resulted, when this vaccine was applied and compared to a number of controls. Importantly, the lifespan of 60% of successfully vaccinated BALB/*c* mice (five of eight) was tripled in the absence of any detectable tumor growth up to 11 weeks after tumor cell challenge (40). In addition, breast cancer cells were killed *in vitro* by both tumor-specific CTLs and NK cells. CD8$^+$ T cells isolated from splenocytes of mice immunized with the vaccine encoding pUb-Fra-1/pIL-18 effectively killed D2F2 breast cancer cells *in vitro* in a ^{51}Cr-release assay. In contrast, such T cells isolated from control animals were ineffective. CTL-mediated killing was specific, because syngeneic prostate cancer-target cells (RM-2) were not lysed. Furthermore, the CD8$^+$ T-cell-mediated tumor cell lysis was MHC class I antigen-restricted, because addition of anti-H2K^d/H-2D^d antibody abrogated tumor cell lysis. NK cells were also effective in killing D2F2 tumor cells in an assay against Yac-1-target cells in contrast to control immunizations, which were ineffective (40).

Immunological mechanisms induced by the vaccine

Interactions between IL-18 and active Th1 and NK cells were found to be critical for achieving both optimal antibody-specific T-cell and NK-cell responses. The pUb-Fra-1/pIL-18 vaccine or pIL-18 alone upregulated the expression of the respective T- and NK-cell markers. This activity was evident from fluorescence-activated cell sorting (FACS) analyses indicating a marked increase over the empty vector control in expression of CD25 (10.4%), the high affinity IL-2 receptor α-chain, CD69 on early T-cell activation antigen (14.1%), CD28 (12.2%), and CD11a (17.1%), all of which are important for initial interaction between T cells and DCs, as well as regular T-cell markers CD4 (14.5%) and CD8 (16.1%). As it is known that NK cells can also play a role in anti-tumor immune responses, we tested spleen cells from immunized and control mice with anti-DX-5 antibody and found DX5 expression, important for NK cytotoxicity, to be markedly increased from 2 to 35.3% (40).

An increase found in costimulatory molecules on DCs was significant, as it is well known that T-cell activation depends on upregulated expression of costimulatory molecules CD80 and CD86 on DCs to achieve optimal ligation with CD28 on activated T cells. In fact, FACS analysis of splenocytes from successfully immunized mice and controls indicated that expression of CD80 and CD86 was markedly upregulated by the vaccine on CD11c$^+$ DCs by 10 and 9.5%, respectively (40). The activation of T cells by the pFra-1/pIL-18 vaccine was further demonstrated by their increased secretion of IFN-γ and IL-2. The release of these two proinflammatory cytokines from T cells indicates their activation in secondary lymphoid tissues. An analysis for these cytokines, both intracellularly with flow cytometry or at the single cell level by ELISPOT, indicated that vaccination with the pUb-Fra-1/pIL-18 plasmid and a subsequent challenge with D2F2 tumor cells resulted in marked increases of IFN-γ and IL-2 release over that induced by controls (40).

Suppression of angiogenesis

Anti-angiogenic effects were evaluated 2 weeks after the last oral vaccination with the pUb-Fra-1-pIL-18 DNA plasmid in mice, injected subcutaneously in the sternal region with growth factor reduced Matrigel containing murine FGF2 and

irradiated D2F2 tumor cells. Six days later, the endothelium of these animals was stained by intravenous injection of fluorescent *Bandeiraea simplicifolica* lectin I, and 30 min thereafter, Matrigel plugs were excised for macroscopic evaluation. Lectin-FITC was then extracted from these plugs and quantified by fluorimetry. Distinct suppression of angiogenesis induced by the DNA vaccine was demonstrated in this Matrigel assay by a marked decrease in vascularization. This outcome was evident from evaluation of fluorescence after *in vivo* staining of endothelium of mice with FITC-conjugated lectin. In fact, such differences were visible macroscopically in representative Matrigel plugs removed from vaccinated mice 6 days after their injection. Suppression of angiogenesis was clearly evident from FITC-lectin staining, indicating decreased vascularization in Matrigel plugs after vaccination with the pUb-Fra-1/pIL-18 vaccine and to a lesser extent with pIL-18 but not with control vaccines (40).

Conclusions and future perspectives

The DNA-based cancer vaccines described here all induce a T-cell-mediated immune response sufficiently robust to break peripheral T-cell tolerance against several self-antigens, resulting in the effective generation of tumor-protective immunity. This immunological effector mechanism appears to be dominant, irrespective whether the vaccine targets are the human oncofetal antigen CEA, melanoma antigen peptide epitopes $gp100_{25-33}$ or TRP-2 $_{181-188}$, VEGFR-2 (FLK-1), or transcription factor Fra-1. A common denominator for all these DNA-based vaccines is their oral delivery vehicle of attenuated *S. typhimurium* which targets them to secondary lymphoid organs considered to be optimal for induction of anti-tumor immune responses induced by DNA vaccines (46). The addition of a recombinant antibody fusion protein delivering IL-2 to the tumor microenvironment further boosted the anti-tumor effect of a CEA-based DNA vaccine, particularly when combined with the co-expression of CD40LT. Co-expression of polyubiquitin, which targets proteins to the proteasome, has been effective for optimal antigen processing, particularly for the melanoma minigene vaccine (19, 20). Robust suppression of angiogenesis in the tumor vasculature proved especially effective for the DNA vaccine targeting FLK-1, overexpressed on proliferating endothelial cells (37), and to some extent for the Fra-1-based vaccine, particularly due to its co-expression of IL-18 (40). The CEA- and Fra-1/IL-18-based DNA vaccines were primarily effective in inducing tumor protection in a prophylactic setting, whereas the FLK-1 vaccine was also effective against spontaneous and established tumor metastases in a therapeutic setting. This particular vaccine has several advantages by specifically targeting $CD8^+$ T cells to proliferating endothelial cells in the tumor vasculature rather than directly to tumor cells. Firstly, endothelial cells are genetically stable and do not downregulate MHC class I and II antigens, an event that frequently occurs in solid tumors and severely impairs T-cell-mediated anti-tumor responses (47). In addition, immune suppression triggered by tumor cells at the cellular level can also be avoided by this approach. Secondly, the therapeutic target is tumor-independent, thus killing of proliferating endothelial cells in the tumor microenvironment can be effective against a variety of malignancies. Furthermore, proliferating endothelial cells are readily available to lymphocytes in the bloodstream. Consequently, the target tissue can be reached unimpaired by anatomical barriers such as the blood–brain barrier or encapsulation of tumor tissues (48).

Future perspectives certainly include DNA-based cancer vaccines which not only suppress tumor angiogenesis but also induce effective tumor cell apoptosis. One such candidate DNA vaccine is currently under investigation in the authors' laboratory and targets the inhibition of apoptosis protein surviving which is overexpressed in both tumor cells and proliferating endothelial cells in the tumor vasculature (49). In order to effectively treat established human cancer, it is most likely necessary to use such a novel DNA vaccine possibly in combination with chronically administered chemotherapy at metronomic dose levels, i.e. lower than maximum tolerated dose (50). This combined approach could be of considerable interest, particularly because Kerbel *et al.* (51) reported recently that thrombospondin-1, a mediator of anti-angiogenic effects, is induced by prolonged *in vitro* exposure of endothelial cells to metronomic doses of chemotherapy. Such combined approaches may ultimately lead to the rational design of novel and effective modalities for the treatment of cancer. However, the holy grail of DNA-based cancer vaccines remains the ultimate immunoprevention of cancer, which at this time still remains a distant but plausible prospect.

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DNA Vaccines Suppress Angiogenesis and Protect Against Growth of Breast Cancer Metastases

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Abstract. Two novel oral DNA-based vaccines provide immune protection against breast cancer in mouse model systems. These vaccines are delivered by attenuated *Salmonella typhimurium* to secondary lymphoid organs and are directed against novel targets such as transcription factor Fos-related antigen 1 (Fra-1) and endoglin (CD105). Both vaccines elicit suppression of angiogenesis in the breast tumor vasculature and break peripheral tolerance by eliciting potent cell-mediated protective immunity against these tumor self-antigens resulting in effective suppression of breast tumor growth and metastasis.

INTRODUCTION

A recent overview by Maurice R. Hilleman on the needs and realities for developing new and improved vaccines in the 21st century ends with the conclusion that "the development of multispecific vaccines that can be given orally, transdermally or mucosally may be the holy grail of future endeavor" [1]. In fact, our efforts, outlined in this chapter to develop novel DNA-based vaccines against breast cancer are very much guided by the principle of multifunctional DNA vaccines that are delivered orally to secondary lymphoid tissues by attenuated *Salmonella typhimurium*. Our goal is to induce the most effective and long-lived protective immune response possible against unique targets expressed by breast tumors and/or proliferating endothelial cells in their vasculature. In contrast to immunotherapy which relies on the administration of preformed effector mechanisms and is ideally suited to the treatment of established tumors, cancer vaccines are developed with an eye on prevention [2]. Based on successes achieved by vaccines in preventing infectious disease, our current efforts in cancer vaccines focus on the definition of unique vaccine targets not only expressed by tumors but also by their vasculature or stroma, and on the development of immunization protocols that will be highly effective in priming the immune system to eliminate cancer before it manifests itself clinically or in delaying/preventing cancer recurrence. However, this entire approach is handicapped by the very fact that most tumor-associated antigens are poorly immunogenic self-antigens that necessitate the breaking of immunological tolerance against them by suitably designed immunological strategies, including powerful vaccine adjuvants. Among such adjuvants are cytokines or chemokines which we co-express as secretory components in our DNA vaccines to suitably enhance the activity of both, antigen presenting cells (APCs) and immune effector cells and thereby induce an optimal anti-tumor immune response. Here, we highlight two novel approaches for breast cancer vaccines based on results from our recent basic and pre-clinical studies that are providing support for preventive DNA-based breast cancer vaccines. First, a transcription factor Fos-related antigen 1 (Fra-1), overexpressed by D2F2 murine breast cancer cells, was demonstrated to be an effective target for a DNA-based breast cancer vaccine, which suppressed angiogenesis as well as tumor growth and dissemination, especially when co-expressed with secretory murine IL-18. Sec-

ond, endoglin (CD105) a binding protein of the transforming growth factor β receptor complex, overexpressed by proliferating endothelial cells in the D2F2 breast tumor vasculature, proved to be an excellent target for a DNA vaccine that was effective in suppressing tumor angiogenesis and subsequently tumor growth and dissemination in animal models.

Challenges for effective DNA-based cancer vaccines arise mainly from the poor immunogenicity of tumor-associated self-antigens. In fact, this necessitates the careful selection of the most effective target antigen; choosing the right delivery vehicle for DNA vaccines; and to design effective immunization protocols, including the most optimal vaccine adjuvants for the generation of a robust tumor protective immune response.

Selection of Effective Target Antigens

The major advantage which cancer immunotherapy has over other forms of therapy is its unique specificity whereby the immune response can recognize epitopes expressed by tumor cells or their microenvironment and target those cells for destruction without harming normal cells [3]. Research efforts in tumor immunology made during the last 20 years took full advantage of this specificity and identified a number of molecularly-defined tumor antigens which in pre-clinical studies elicited tumor-specific immunity and established long-term memory without inducing autoimmunity [3]. For example, breast cancer vaccine targets are composed of a number of well characterized epitopes such as HER2/Neu [4], mucin 1 [5], MAGE 3 [6] or carcinoembryonic antigen (CEA) [7], to name just a few. We selected two quite different antigens as respective targets for our breast cancer vaccines, namely transcription factor Fos-related antigen 1 (Fra-1) and endoglin (CD105) a binding protein of the TGF-β1, and TGF-β3 receptor complex, overexpressed by proliferating endothelial cells in the breast tumor vasculature but not expressed by breast tumor cells.

Our rationale for selecting Fra-1 as a vaccine target was based on several considerations. First, a limited number of transcription factors are generally overactive in most cancer cells which makes them appropriate targets for anticancer drugs, provided selective inhibition of transcription can be applied rather than general inhibition which is expected to be too toxic [8]. In fact, rather than selecting specific inhibitors of a transcription factor, we chose Fra-1, belonging to the transcription factor activating protein-1 (AP-1) family, since it defines tumor progression and regulates breast cancer cell invasion and growth as well as resistance to anti-estrogens. In addition, Fra-1 is overexpressed by many human and mouse epithelial carcinoma cells, including breast cancer cells [9]. This overexpression of Fra-1 greatly influences these cells' morphology and motility, correlates with their transformation to a more invasive phenotype and is specifically associated with highly invasive breast cancer cells. These findings suggest Fra-1 to be a potentially useful target for active immunization against breast cancer [10].

The selection of endoglin (CD105) as a DNA vaccine target was based primarily on its overexpression by proliferating endothelial cells in the breast tumor vasculature which facilitates the induction of T cell-mediated suppression of tumor angiogenesis. This was already successfully demonstrated by us for a DNA vaccine against murine VEGF receptor-2 (FLK-1) [11]. Similar to endoglin, this receptor typosine kinase is also upregulated by genetically stable proliferating endothelial cells in the tumor vasculature, but not expressed by genetically unstable, frequently mutating tumor cells. In fact, attacking the tumor's vascular supply to inhibit tumor growth, which was pioneered by Folkman and colleagues [12–15], is an attractive strategy for several reasons. First, angiogenesis is a rate-limiting step in the development of tumors since tumor growth is generally limited to 1–2 mm^3 in the absence of a blood supply [16,17]. Beyond this minimum size, tumors frequently become necrotic and apoptotic under such circumstances [18]. Second, the suppression of tumor-associated angiogenesis is a physiological host mechanism and should not lead to the development of resistance. Third, each tumor capillary has the potential to supply many tumor cells, so that targeting the tumor vasculature potentiates the antitumor effect. Fourth, direct contact of the vasculature with the circulation leads to efficient access of therapeutic agents [15]. Importantly, targeting of genetically stable proliferating endothelial cells in the tumor vasculature does not downregulate MHC class I and II antigens – an event that frequently occurs in most solid tumors, including breast cancer, which severely impairs T cell-mediated antitumor responses that are of paramount importance for DNA vaccine strategies. Additional advantages of this approach include the avoidance of immune suppression triggered by tumor cells at the cellular level; tumor independence of the therapeutic target, making this approach effective against a whole variety of malignancies; and ready availability of proliferating endothelial cells to lymphocytes in the circulation making it possible to reach target tissue unimpaired by anatomical barriers such as the blood-brain barrier or encapsulated tumor tissues [19].

Choosing a DNA Vaccine Delivery Vehicle

The potential use of attenuated strains of *Salmonella typhimurium* as a DNA vaccine carrier is based on initial findings by Hoiseth and Stocker [20] that an aromatic-dependent auxotrophic mutant of *Salmonella typhimurium* is non-virulent and effective as a live vaccine [21]. Later work with these attenuated (AroA) bacteria indicated that they could be used for oral somatic transgene vaccination [22] and to trigger the elicitation of antigen-specific humoral, T helper and cytotoxic responses against β-galactosidase, a model antigen [23]. Importantly, since professional antigen-presenting cells (APCs) play a key role in the induction of effective immune responses evoked by vaccination with plasmid DNA, the use of attenuated intracellular bacteria as delivery vehicle has the potential to efficiently target DNA vaccines to professional APCs.

We first demonstrated that the attenuated (AroA-) strain SL7207 of *Salmonella typhimurium*, made available by B.A.D. Stocker (Stanford University), was an effective carrier for oral delivery by gavage of an autologous DNA vaccine which effectively protected against challenge with murine melanoma cells. In fact, this vaccine broke peripheral T cell tolerance toward murine melanoma self antigens gp100 and TRP-2 containing the murine ubiquitin gene fused to minigenes encoding peptide epitopes $gp100_{25-33}$ and $TRP\text{-}2_{181-188}$ and induced a robust, tumor-specific $CD8^+$ T cell response resulting in suppression of melanoma tumor growth [24]. Following gavage, these live, attenuated bacteria transport the DNA through the small intestine and then through the M cells that cover the Peyer's patches of the gut. From there the attenuated bacteria enter APCs such as dendritic cells and macrophages, which are plentiful in this secondary lymphoid tissue, where they die because of their AroA- mutation, liberating multiple copies of the DNA inside these phagocytes. There, the DNA is transcribed to protein which is then processed in the proteasomes of these APCs. This is followed by the formation of peptide-MHC class I antigen complexes which are ultimately presented by the APCs to the T cell receptor of naïve T cells. This entire process is depicted schematically in Fig. 1. We have successfully used attenuated *Salmonella typhimurium* as a carrier for several of our DNA vaccines encoding the human CEA gene effective in eliciting potent $CD8^+$ T cell immunity in CEA-transgenic mice that eradicated growth and metastases of colon [25] and non-small cell lung cancer [26]. This same approach was also used successfully to deliver plasmids encoding genes for VEGF-receptor 2 (FLK-1) to Peyer's patches resulting in a robust T cell mediated immune response against proliferating endothelial cells in the tumor vasculature in three different tumor models. Thislead ultimately to effective suppression of angiogenesis and subsequent eradication of tumor metastases in both prophylactic and therapeutic settings [11].

Our rationale for delivering DNA vaccines orally by gavage with attenuated *Salmonella typhimurium* to Peyer's patches was strongly supported by results of a recent study by Maloy et al. [27] clearly indicating that intralymphatic immunization is the most effective means to strongly enhance DNA vaccination. This was evident from a comparison of conventional routes of immunization given i.d., i.m. or i.spl. with intralymphatic immunizations of DNA encoding CTL epitopes of the highly immunogenic LCMV glycoprotein. In this case, direct injection of the DNA into a peripheral lymph node enhanced immunogenicity by 100 to 1000-fold, inducing strong and biologically relevant $CD8^+$ cytotoxic T lymphocyte responses.

We successfully transferred the plasmid encoding the Fra-1 gene by electroporation into doubly attenuated (dam-,AroA-) *Salmonella typhimurium* and then administered it as an oral vaccine by gavage to BALB/c mice. Here, two constructs were made based on the pIRES vector. As depicted in Fig. 2(A), the first, pUb-Fra-1, was comprised of polyubiquitinated, full-length murine Fra-1. The second, pIL-18, contained murine IL-18 with an Ig kappa leader sequence for secretion purposes. The empty vector with or without ubiquitin served as a control. By way of explanation, polyubiquitination has been used for many of our DNA vaccines, particularly since we found in one of our initial studies that the presence of ubiquitin upstream of a DNA minigene encoding melanoma antigens proved to be essential for achieving tumor-protective immunity [24]. Based on a vast body of literature on the role of ubiquitin in protein processing in the proteasome [28], we assumed that this molecule was essential for optimizing antigen processing and ultimately effective antigen presentation. Although ubiquitination does not invariably enhance $CD8^+$ T cell responses, our work and reports by other investigators confirmed the important role played by ubiquitination in the MHC class I antigen presentation pathway [24].

As far as our Fra-1 vaccine is concerned, we demonstrated protein expression of pUb-Fra-1 and pIL-18 by transient transfection of each vector into COS-7 cells and by performing Western blots of the respective cell lysates (pUb-Fra-1 or pIL-18) and supernatant (pIL-

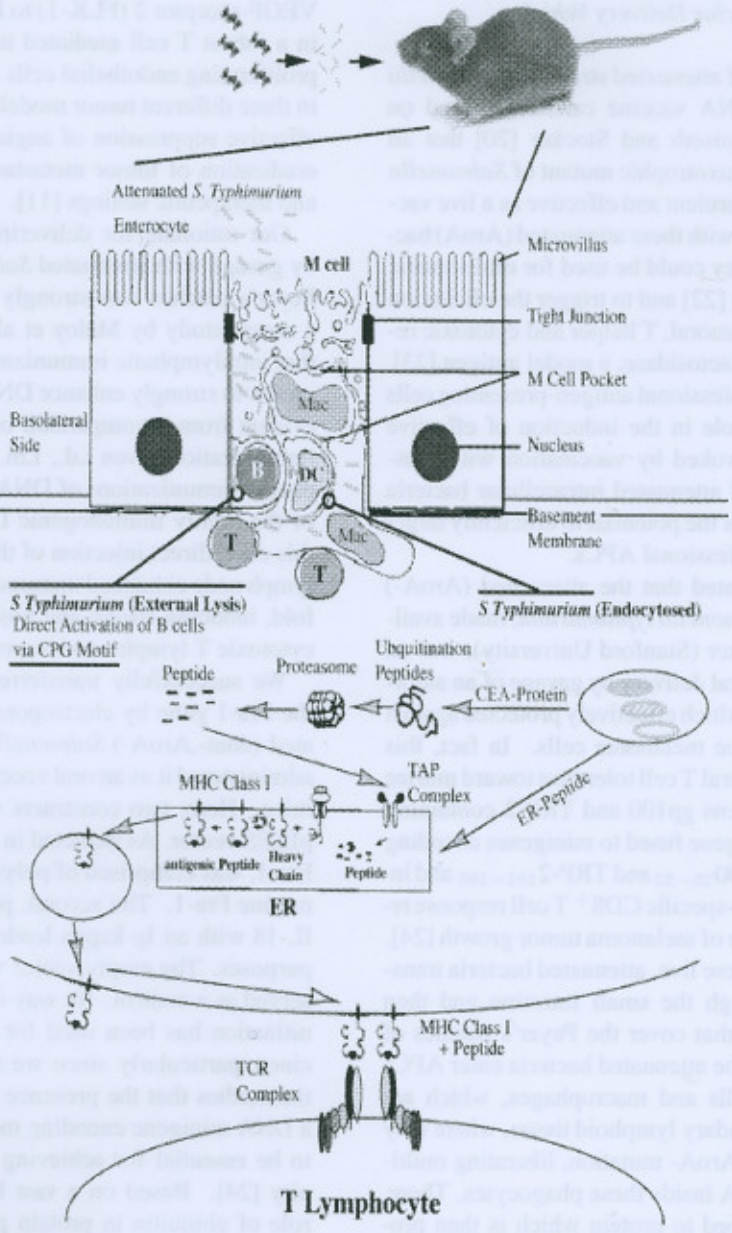

Fig. 1. Schematic diagram of mechanisms of action elicited by DNA vaccines delivered by attenuated *Salmonella typhimurium* to Peyer's patches.

18) with anti-Fra-1 and anti-IL-18 Ab, respectively. As shown in Fig. 2(A), all constructs produced protein of the expected molecular mass with IL-18 being expressed in its active form at 18 KDa (Fig. 2(A), lane 2) and Fra-1 as a 46 KDa protein (Fig. 2(A), lane 1). Protein expression of pIL-18 was also detected in the culture supernatant of transfected cells (Fig. 2(A), lane 3). Importantly, the biofunctional activity of IL-18 was clearly demonstrated by ELISA in supernatants of cells transfected with pIL-18 (Fig. 2(B)).

Furthermore, the data depicted in Fig. 2(C) demonstrate that the attenuated *Salmonella typhimurium* successfully transferred expression vectors to mouse Peyer's patches. Thus, DNA encoding pUb-Fra-1 and pIL-18 was successfully released from the attenuated bacteria and entered Peyer's patches in the small intestine (Fig. 2(C). The DNA was subsequently transcribed by APCs, processed in the proteasome, and presented as

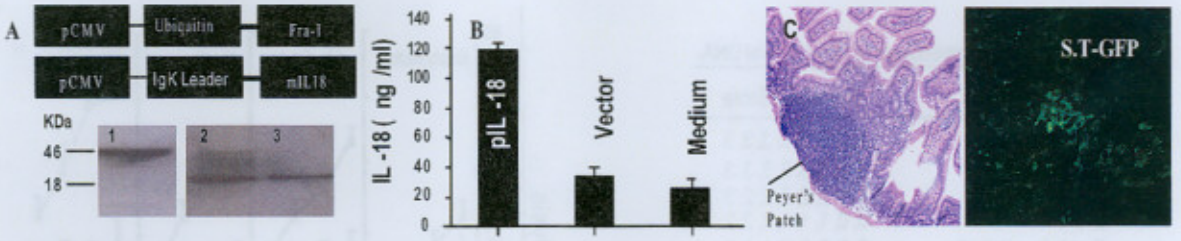

Fig. 2. Vector construction map, protein expression, bioactivity, and targeting of expression constructs. (A) The coding sequence of full-length, murine Fra-1, fused with polyubiquitin at the N terminus, was inserted into the pIRES plasmid (pUb-Fra-1). A second plasmid, pIL-18, contained the entire coding sequence of murine IL-18 with an lgk leader sequence. Protein expression by pUb-Fra-1 and pIL-18 was demonstrated by Western blotting. Blots are shown for either pUb- Fra-1 (lane 1) or pIL-18 (lane 2) and of culture supernatant from pIL-18- transfected COS-7 cells (lane 3). (B) Bioactivity of IL-18 (ng/ml) determined by ELISA in supernatants of KG-1 lymphoma cells that were transfected with pIL-18. The error bars indicate mean standard deviation of multiple assays. (C) Expression of EGFP activity in Peyer's patches was determined in mice immunized with 10^8 CFU of Aro-, dam- bacteria transformed with pEGFP (S.T- GFP) by gavage. Fluorescence expression of EGFP was detected by confocol microscopy (Right). Hematoxylin/eosin staining of mouse Peyer's patches is shown (Left).

MHC-peptide complexes to T cells. To this end, mice were administered by gavage 1×10^8 CFU of dam-, AroA- attenuated *S. typhimurium*. After 24 h these animals were killed and biopsies were collected from the thoroughly washed small intestine. In fact, the doubly attenuated bacteria harboring EGFP (S.T-GFP) exhibited strong EGFP fluorescence (Fig. 2(C)). This finding suggested not only that such bacteria can transfer the target gene to Peyer's patches, but also that plasmids encoding each individual gene can successfully express their respective proteins. Importantly, these doubly attenuated bacteria do not survive very long because neither EGFP activity nor live bacteria could be detected in immunized animals after 72 hours (data not shown). However, EGFP expression was detected in adherent cells, most likely APCs, such as dendritic cells and macrophages from Peyer's patches after oral administration of S. typhimurium harboring the eukaryotic EGFP expression plasmid. Taken together, these findings indicate that both plasmid transfer to and protein expression in eukaryotic cells did take place.

IMMUNIZATION WITH BREAST CANCER VACCINES

DNA Vaccine Encoding Fra-1/IL-18

There is a rationale for the development of prophylactic cancer vaccines since considerable data from experimental systems have shown that immunity can be activated to prevent tumors. Thus, there is a strong rationale for prevention since in such a setting one deals with an immune system which is unimpaired by immune suppression induced by tumors and/or treatment. Neither is there tolerance to tumor antigens that were confronted in the absence of appropriate antigen presentation and costimulatory signals. In such a setting, the use of overexpressed growth factor receptors or transcription factor related antigens yields rational targets for specific immune prevention, also in individuals whose tumors were eradicated by standard therapies. The rationale for developing such prophylactic breast cancer vaccines has thus guided our research efforts described in this article.

Induction of Tumor-Specific Protective Immunity

We tested the hypothesis that an orally administered DNA vaccine encoding murine, pUb-Fra-1 together with secretory pIL-18 (pUb-Fra-1/pIL-18), carried by attenuated *Salmonella typhimurium* can induce protective immunity against s.c. tumors and experimental pulmonary metastases of D2F2 breast carcinoma. Thus, Fig. 3(A) shows marked suppression of disseminated pulmonary metastases in BALB/c mice challenged 1 week after the third vaccination at 2 week intervals with pUb-Fra-1/pIL-18 by an i.v. injection of D2F2 tumor cells. A marked increase in tumor volume of s.c. D2F2 was also evident when this vaccine was applied and compared to a number of controls (Fig. 3(B)). Importantly, the life span of 60% of successfully vaccinated BALB/c mice (5/8) was tripled in the absence of any detectable tumor growth up to 11 weeks after tumor cell challenge (Fig. 3(C)).

Breast Cancer Cells are Killed in vitro by Tumor-Specific CTLs and NK Cells

The data shown in Fig. 4 indicate that $CD8^+$ T cells isolated from splenocytes of mice immunized with the vaccine encoding pUb-Fra-1/pIL-18 effectively killed D2F2 breast cancer cells *in vitro* in a ^{51}Cr-release assay.

A

Suppression of lung metastases of D2F2 by oral DNA vaccine

Treatment groups	Metastatic Score
A. Empty vector	2, 3, 3, 3, 3, 3, 3, 3
B. Pub	3, 3, 3, 3, 3, 3, 3, 3
C. pUb-Fra-1	1, 1, 2, 2, 2, 2, 2, 3
D. pIL-18	0, 0, 1, 1, 1, 2, 2, 2
E. pUb-Fra-1/pIL-18	0, 0, 0, 0, 0, 1, 1, 2

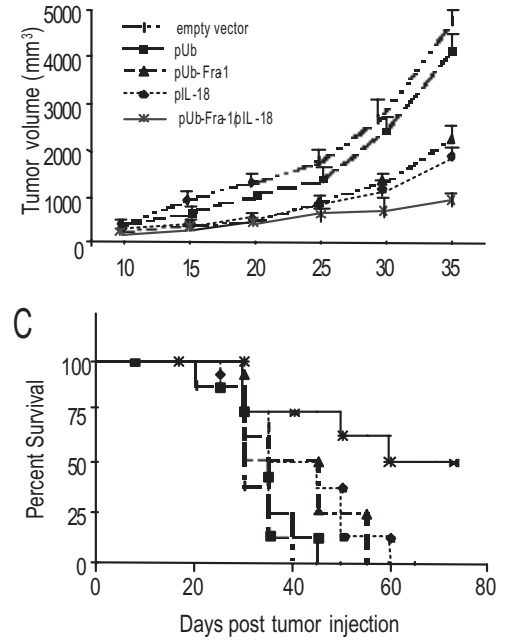

Fig. 3. Effect of the pUb-Fra-1/pIL-18 based DNA vaccine on primary tumor growth and metastases. Each experimental group ($n = 8$) of BALB/c mice was vaccinated by gavage. (A) Suppression of experimental pulmonary metastases of D2F2 breast carcinoma. Results are expressed as metastatic score, i.e. percentage of lung surface covered by fused tumor foci. (B) Tumor growth was analyzed in mice challenged s.c. with 1×10^6 D2F2 tumor cells 1 wk after the last vaccination in each treatment or control group. (C) Survival curves represent results for 8 mice in each of the treatment and control groups. Surviving mice were tumor free unless otherwise stated.

In contrast, such T cells isolated from control animals were ineffective. Thus CTL-mediated killing was specific since syngeneic prostate cancer target cells (RM-2) were not lysed (data not shown). The $CD8^+$ T cell-mediated tumor cell lysis was MHC class I antigen-restricted since addition of anti-$H2K^d$/$H2D^d$ Absabrogated tumor cell lysis (Fig. 4). NK cells were also effective in killing D2F2 tumor cells in an assay against Yac-1 target cells in contrast to control immunizations which were ineffective (Fig. 4).

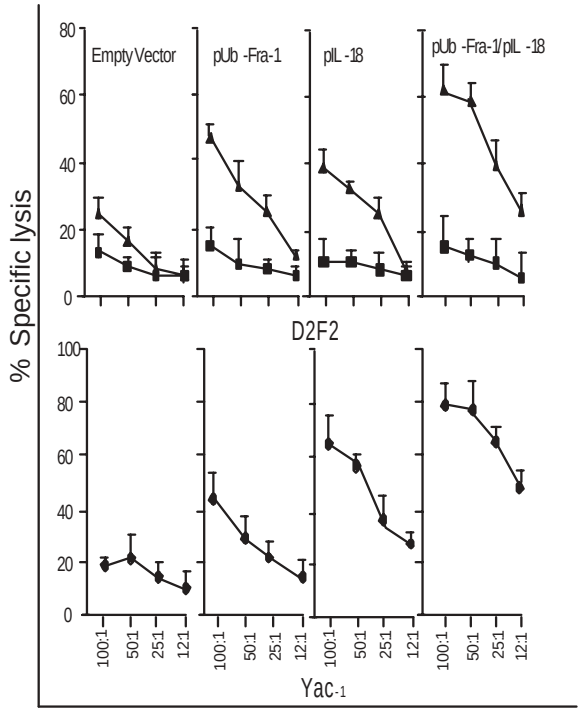

Fig. 4. Cytotoxicity induced by $CD8^+$ T and NK cells. Splenocytes were isolated from BALB/c mice after vaccination with experimental or control DNA vaccines 2 wk after challenge with D2F2 tumor cells and analyzed for their cytotoxic activity in a ^{51}Cr-release assay at different effector-to-target cell ratios. (Upper) Specific lysis mediated by $CD8^+$ T cells against D2F2 target cells (▲), which was blocked by an anti-MHC-class I Ab ($H\text{-}2K^d$/$H\text{-}2D^d$)(■). (Lower) Lysis mediated by NK cells (●) against Yac-1 target cells. Each value shown represents the mean of 8 animals.

Specific Activation of T, NK and DCs by the DNA Vaccine

We could demonstrate that interactions between IL-18 and active T helper 1 and NK cells are critical for achieving both optimal Ag-specific T cell and NK cell responses. Thus, the pUb-Fra-1/pIL-18 vaccine or pIL-18 alone up-regulated the expression of the respective T and NK cell markers. This was evident from FACS analyses indicating a marked increase over the empty vector control in expression of CD25 (10.4%), the high affinity IL-2 receptor alpha chain, CD69 on early T cell activation antigen (14.1%), CD28 (12.2%) and CD11a (17.1%), all of which are important for initial interaction between T-and dendritic cells, as well as regular T cell markers $CD4^+$ (14.5%) and $CD8^+$ (16.1%). Since it is known that NK cells can also play a role in antitumor immune responses (29) we tested spleen cells from immunized and control mice with anti-DX-5Ab and found DX5 expression, important for NK cytotoxicity, to be markedly increased from 2% to 35.3% [10].

Increase of Costimulatory Molecules on DCs

It is well known that T cell activation depends on upregulated expression of costimulatory molecules CD80 and CD86 on DCs to achieve optimal ligation with CD28 on activated T cells. Thus, we applied fluorescence activated cell sorting analysis (FACS) of splenocytes from successfully immunized mice and controls and found that expression of CD80 and CD86 was markedly upregulated by the vaccine on $CD11c^+$ DCs by 10% and 9.5%, respectively [10].

Activation of T cells is Indicated by Increased Secretion of IFN-γ and IL-2

We analyzed for the release of these two proinflammatory cytokines from T cells since this is a well known indication of their activation in secondary lymphoid tissues. When analyzing for these cytokines both intracellularly with flow cytometry or at the single cell level by ELISPOT we found that vaccination with the pUb-Fra-1/pIL-18 plasmid and a subsequent challenge with D2F2 tumor cells resulted in marked increases of IFN-γ and IL-2 release over that induced by controls [10].

The pUb-Fra-1/IL-18 DNA Vaccine Suppresses Angiogenesis

We demonstrated distinct suppression of angiogenesis induced by our DNA vaccine in a Matrigel assay. Figure 5 shows a marked decrease in vascularization. This was evident from evaluation of fluorescence after *in vivo* staining of endothelium of mice with FITC-conjugated lectin. As shown in Fig. 5, such differences were visible macroscopically in representative Matrigel plugs removed from vaccinated mice 6 days after their injection. Suppression of angiogenesis is clearly demonstrated by FITC-lectin staining as shown by decreased vascularization in Matrigel plugs after vaccination with pUb-Fra-1/pIL-18 and to a lesser extent with pIL-18, but not with control vaccines [10].

DNA Vaccine Encoding Endoglin

Endoglin (CD105) which is part of the TGF-β_1/TGF-β_3 receptor complex is overexpressed by proliferating endothelial cells in the D2F2 breast tumor vasculature, but not expressed by D2F2 tumor cells. Therefore, this antigen provides an effective target for a DNA vaccine encoding it which, in turn, induces a cytotoxic T cell mediated immune response that can destroy these endothelial cells and thereby suppress angiogenesis in the tumor cell vasculature resulting in suppression of breast tumor growth. This approach is in principle similar to the one we reported previously for a DNA vaccine encoding the murine vascular endothelial growth factor receptor-2 (FLK-1) which is also overexpressed on proliferating endothelial cells in the tumor vasculature, but not expressed by tumor cells. In this case, we could demonstrate a $CD8^+$ T cell mediated immune response resulting in marked suppression of tumor angiogenesis in three mouse tumor models which resulted in eradication of tumor growth and metastases in both prophylactic and therapeutic settings [11].

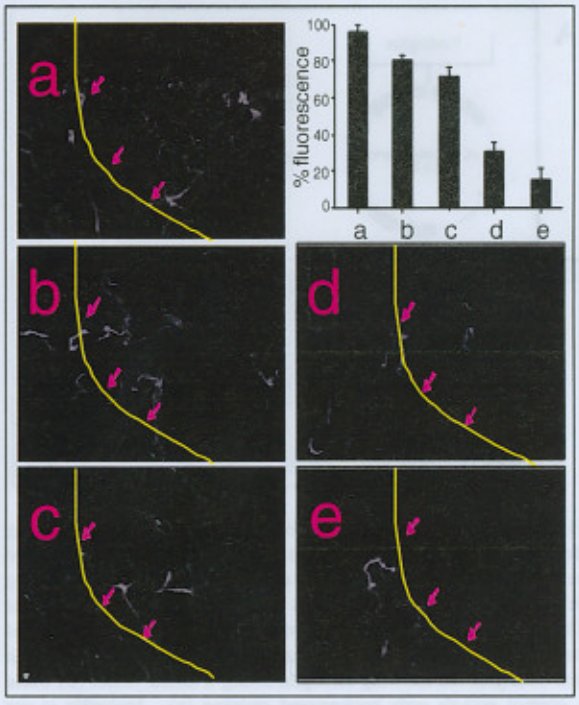

Fig. 5. Suppression of tumor angiogenesis. Antiangiogenesis was determined by the Matrigel assay. Quantification of vessel growth and staining of endothelium was determined by fluorimetry or confocal microscopy, respectively, using FITC- labeled Isolectin B4. The line and arrows (a-e) indicate the inside borders of the Matrigel plug. Matrigel was implanted into mice vaccinated with empty vector (a), pUb (b), pUb-Fra-1 (c), pIL-18 (d), or pUb-Fra-1/pIL-18 (e). The average fluorescence of Matrigel plugs from each group of mice is depicted by the bar graphs ($n = 4$; mean $\pm$ SD). Comparison of control groups (a and b) with treatment groups (d and e) indicated statistical significance ($P < 0.05$).

For all the reasons that were cited above under "Selection of Target Antigens for DNA Vaccines", the genetically stable endothelial cells in the tumor vasculature offer a far better target for cytotoxic T cells than the genetically unstable, frequently mutating tumor cells which often down regulate critical MHC antigens and induce immune suppression at the cellular level. Here, we present some preliminary data providing evidence that a DNA vaccine encoding the entire murine en-

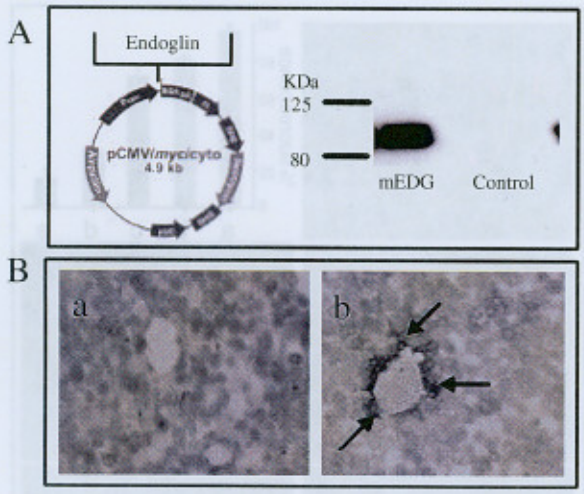

Fig. 6. Schematic of vectors encoding murine endoglin indicating that the entire gene was inserted into the pCMV/myc/cyto expression vector (A). Protein expression was demonstrated by Western blotting of cell lysates from COS-7 cells transfected with endoglin (lane 1)(B). Endoglin was detected by immunohistology in the D2F2 breast tumor vasculature by staining frozen sections of tumor tissue with anti-endoglin Ab showing endoglin lining a blood vessel (b) while a control reveals only background staining (a). Endoglin is not expressed by D2F2 tumor cells.

doglin gene can lower angiogenesis in the tumor vasculature and suppress pulmonary metastases of D2F2 murine breast carcinoma cells in BALB/c mice.

Construction of Endoglin Expression Vector and Protein Expression

The entire gene encoding murine endoglin (CD105) was inserted into the pCMV/myc/cyto expression vector (Fig. 6(A)). Protein expression by endoglin was demonstrated by Western blotting of cell lysates from COS-7 cells transfected with endoglin (lane 1). In Fig. 6(B), endoglin is demonstrated by immunohistology in the D2F2 breast tumor vasculature by staining a frozen section of D2F2 tumor tissue with anti-endoglin Ab showing endoglin lining a blood vessel indicated by arrows while a control tissue reveals only background staining. FACS analyses showed the absence of endoglin expression by D2F2 mouse breast tumor cells (data not shown).

Suppression of D2F2 Pulmonary Breast Carcinoma Metastases

To test the efficacy of the vaccine, BALB/c mice were administered by gavage 10^8 CFU of doubly attenuated *Salmonella typhimurium* (dam-; AroA-) transformed by electroporation with the plasmid encoding endoglin. Vaccination was done by gavage in 100 μl sterile water, 3 times at 2 week intervals in BALB/c mice who received 15 minutes prior to vaccination 50 μl of 7.5% sodium bicarbonate (SB) to neutralize the acid pH of the stomach which is detrimental to the bacteria. One week after the last vaccination, experimental pulmonary metastases were induced by i.v. injection of 5 $\times$ 10^4 D2F2 breast carcinoma cells. The experiment was terminated 28 days after tumor cell challenge and the weight of lungs and extent of tumor foci determined. Figure 7(A) depicting the lungs of mice receiving SB only (a) SB+ empty vector (b) and endoglin vaccine (c), clearly demonstrating that only the vaccine drastically suppressed pulmonary metastases. This is also shown in Fig. 7(B) indicating the weight of the lungs, (normal lung=0.2 g). The extent of metastases is also demonstrated in Fig. 7(C) which shows the metastasis score, i.e. the percent of lung cell surface covered by fused metastases: 0 = none; 1 =< 5%; 2 = 5–50% and 3 = >50% of lung surface covered by metastatic foci. Differences in metastasis scores and lung weights between mice treated with the vaccine and all controls were statistically significant ($P < 0.001$).

Cell-mediated Cytotoxicity Induced by the Endoglin DNA Vaccine Against Murine Endothelial Cells

To assess T cell mediated cytotoxicity, splenocytes of mice ($n = 8$) treated with either the endoglin vaccine or controls treated with SB+empty vector were isolated one week after tumor cell challenge, and analyzed for cytotoxicity in a 4-h ^{51}Cr release assay. Splenocytes from mice immunized with the endoglin vaccine revealed up to 45% cytotoxicity against murine endothelial target cells expressing endoglin when compared to those from controls which showed only background cytotoxic activity of 10% or less ($P < 0.005$).

Suppression of Angiogenesis

The DNA vaccine encoding endoglin, overexpressed by proliferating endothelial cells in the tumor vasculature, but not expressed by D2F2 breast tumor cells, suppressed angiogenesis in the tumor vasculature. This was evident when angiogenesis was measured by the Matrigel assay. In this case, one week after the last of 3 vaccinations with the endoglin vaccine, BALB/c mice were injected s.c. at the abdominal midline with Matrigel, supplemented with basic fibroblast growth factor. Six days later, the endothelium of these mice was stained by i.v. injection of Bandiera simplofica Isolectin conjugated with fluorescein, and 30 minutes thereafter matrigel plugs of 100 μl were evaluated microscopically and then eluted with RIPA lysate buffer

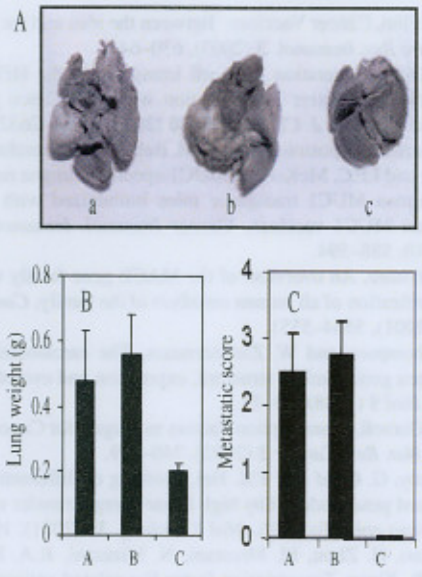

Fig. 7. Experimental lung metastases were induced by i.v. injection of D2F2 cells one week after the last of 3 immunizations administered at 2-week intervals with the endoglin vaccine. The vaccine was administered by gavage to BALB/c mice who received 15 min prior to vaccination 50 μl of 7.5% sodium bicarbonate (SB) to neutralize the acid pH of the stomach. Results are shown in Fig. 7(A) depicting lungs of mice receiving SB only (a), SB + empty vector (b), and the endoglin vaccine (c). Figure 7(B) depicts the average lung weight of experimental mice ($n = 8$) in the same order as shown in Fig. 7(A). Figure 7(C) shows the metastastatic score, ie. the percent of lung cell surface covered by fused metastases: 0=none; 1 =<5%; 2=5–50% and 3=>50%.

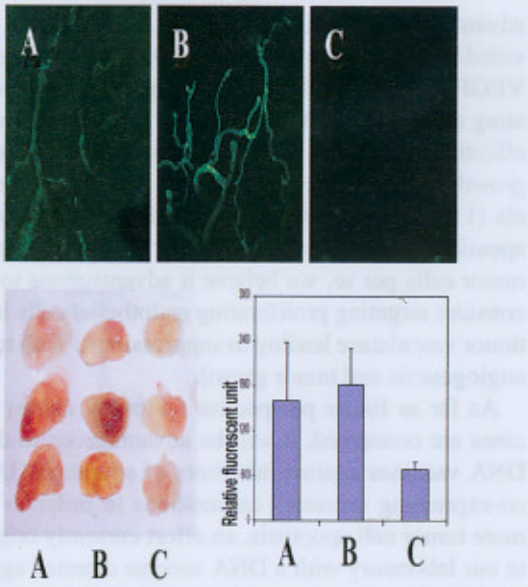

Fig. 8. Suppression of tumor angiogenesis by the DNA based vaccine encoding endoglin. Antiangiogenesis was determined by the Matrigel assay. Quantification of vessel growth and staining of endothelium was determined by fluorimetry or confocal microscopy, respectively, using empty vector (A), SB alone (B), SB+ empty vector (C), SB+endoglin vaccine. The average fluorescence of Matrigel plugs from each group of mice is depicted by the bar graphs ($P < 0.05$)($n = 4$;mean $\pm$ SD).

to measure fluorescence. As depicted in Fig. 8 it is quite evident that the endoglin vaccine effectively suppressed angiogenesis in the tumor vasculature when compared to controls. Quantification of vessel growth and staining of endothelium was determined by fluorimetry or confocal microscopy. The average fluorescence of Matrigel plugs from each experimental group of mice is depicted by the bar graphs (Fig. 8) where differences between the vaccine and control groups were statistically significant ($n = 4$; $P < 0.05$).

CONCLUSIONS AND PERSPECTIVES

Taken together, our data suggest that peripheral T cell tolerance against the Fra-1 transcription factor was broken by a DNA vaccine encoding it, particularly when fused with polyubiquitin and modified by co-transformation with a gene encoding murine IL-18. Furthermore, a robust immune response was mediated by $CD4^+$ T cells, $CD8^+$ T cells and NK cells controlled by upregulation of IFN-γ. Our vaccine design was successful since activation of both T and NK cells was augmented as was that of dendritic cells as indicated by upregulation of CD80 and CD86 costimulatory molecules. We believe that our success in eliciting an active $CD8^+$ T cell mediated tumor protective immune response with a completely autologous DNA vaccine, as well as the induction of antiangiogenesis was aided by our efforts to optimize antigen processing in the proteasome with polyubiquitination [10,11, 24–26]. Also, one of the more critical aspects of DNA vaccine design is the selection of an optimal carrier to deliver a target gene to secondary lymphoid organs, such as Peyer's patches. In this case, the live, attenuated *Salmonella typhimurium* harboring eukaryotic expression plasmids encoding Ag proved to be an effective vehicle for oral vaccine delivery, especially when combined with an adjuvant like IL-18.

Although the data obtained with the DNA vaccine encoding endoglin (CD105) are still preliminary, they suggest that a prophylactic vaccine capable of suppressing angiogenesis in the tumor vasculature could be of value for future clinical application.

As pointed out in this article the targeting of genetically stable proliferating endothelial cells rather than genetically unstable, often mutating tumor cells has its

advantages. The rationale for this approach was provided by our prior findings with a DNA vaccine against VEGF receptor 2 (FLK-1), overexpressed by proliferating endothelial cells in the tumor vasculature, which effectively suppressed angiogenesis and thereby tumor growth and metastasis in several solid tumor models [11]. Although the vast majority of immunotherapeutic regimens for breast cancer is directed against tumor cells per se, we believe it advantageous to also consider targeting proliferating endothelial cells in the tumor vasculature leading to suppression of both tumor angiogenesis and tumor growth.

As far as future perspective for breast cancer vaccines are concerned, it will be advantageous to direct DNA vaccines against inhibitors of apoptosis (IAPs), co-expressing secretory chemokines in order to promote tumor cell apoptosis, an effort currently ongoing in our laboratory with a DNA vaccine directed against the IAP Survivin which is overexpressed by most solid tumor cells. Since this IAP is also overexpressed by proliferating endothelial cells in the tumor vasculature, it is feasible for such a vaccine to both suppress angiogenesis in the tumor vasculature and induce tumor cell apoptosis, thereby concurrently producing a very potent anti-tumor effect. It is anticipated that such future research efforts will further contribute towards the rational design of DNA vaccines for the prevention and treatment of breast cancer in a setting of minimal residual disease.

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Endoglin (CD105) is a Target for an Oral DNA Vaccine against Breast Cancer

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Abstract:

Endoglin (CD105), a co-receptor in the TGF-beta receptor complex, is over-expressed on proliferating endothelial cells in the breast tumor neovasculature and thus offers an attractive target for anti-angiogenic therapy. Here we report the anti-angiogenic/anti-tumor effects achieved in a prophylactic setting with an oral DNA vaccine encoding murine endoglin, carried by double attenuated *Salmonella typhimurium* (*dam*$^-$, *AroA*$^-$) to a secondary lymphoid organ, i.e., Peyer's Patches (PP). We demonstrate that an endoglin vaccine elicited activation of antigen-presenting dendritic cells (DCs), coupled with immune responses mediated by $CD8^+$ T cells against endoglin-positive target cells. Moreover, we observed suppression of angiogenesis only in mice administered with the endoglin vaccine as compared to controls. These data suggest that a $CD8^+$ T cell-mediated immune response induced by this vaccine effectively suppressed dissemination of pulmonary metastases of D2F2 breast carcinoma cells presumably by eliminating proliferating endothelial cells in the tumor vasculature. It is anticipated that vaccine strategies such as this may contribute to future therapies for breast cancer.

Introduction

Angiogenesis, the growth of new capillary blood vessels from preexisting vasculature, is an essential feature of tumor growth and metastasis. In fact, anti-angiogenenic therapy, originally proposed by Folkman more than 30 years ago [10] has become a most attractive concept, receiving ever increasing attention during the last decade [12;14;34;41]. The goal of this approach has been to deliver anti-angiogenic agents to appropriate targets in the tumor vasculature to eliminate or suppress blood supply to tumors, resulting in their ablation or growth suppression without seriously disturbing blood flow to normal tissues [3]. Several approaches have been reported to suppress of murine tumor growth and metastasis through anti-angiogenesis by targeting specific molecules such as vascular endothelial growth factor receptor 2 (VEGF-R2) [31]. Our laboratory also demonstrated that a survivin-based oral DNA vaccine, coexpressing the chemokine CCL21, induced effective suppression of angiogenesis by triggering potent CTLs against tumor cells and proliferating endothelial cells expressing survivin, resulting in the suppression or eradication of metastases in a murine tumor models [47].

Endoglin (CD105) is a 180kDa homodimeric transmembrane glycoprotein, primarily expressed on endothelial cells. It acts as an auxiliary protein that interacts with the ligand-binding receptors of multiple members of the transforming growth factor beta (TGF- β) superfamily [4]. Studies have suggested that endoglin offers an excellent target for anti-angiogenic therapy since it is over-expressed on proliferating endothelial cells in blood vessels of tumor tissue. In fact, endoglin and its ligand, TGF-β, are significant modulators

of angiogenesis [16;19]. Moreover, endoglin expression on endothelial cells is up-regulated by TGF-β and hypoxic conditions [40]. In solid tumors such as breast carcinoma, endoglin is almost exclusively expressed on endothelial cells of both peri- and intratumoral blood vessels and on tumor stromal components [5]. Furthermore, a monoclonal endoglin Ab was reported to react with small and immature tumor blood vessels in prostate and breast cancer [46], and to strongly stain endothelial cells, but not smooth muscle cells associated with blood vessels within all tumor lesions investigated [7]. In addition, quantifying tumor microvessel density with this same Ab also proved to be an independent prognostic parameter for survival of colorectal cancer patients [45]. Taken together, these data suggest the involvement of endoglin in tumor angiogenesis and point it as a candidate for vascular targeting in tumor therapy, especially since endoglin is not detectable in blood vessels within normal tissues [18;20;38]. In fact, Seon et.al successfully applied an anti-human endoglin immunotoxin to inhibit growth of subcutaneous MCF7 human breast carcinoma in SCID mice [42]. Recently, synergy was demonstrated between endoglin mAbs and TGF- β in growth suppression of human endothelial cells *in vitro*, suggesting that TGF-β plays a key role by synergistically enhancing the anti-angiogenic activity of such antibodies [43]. In addition, endoglin-based xenogeneic vaccination was shown to effectively elicit both protective and therapeutic anti-tumor immunity in several mouse tumor models [44]. Preliminary data obtained in our laboratory suggested that a DNA vaccine encoding the entire murine endoglin gene suppressed angiogenesis and pulmonary metastases of murine breast carcinoma [29].

Here, we demonstrate that a DNA vaccine encoding murine endoglin was delivered orally by attenuated *Salmonella typhimurium* to secondary lymphoid organs such as Peyer's patches (PPs). This vaccine overcame peripheral T cell tolerance and induced a robust $CD8^+$ T cell mediated immune response that inhibited angiogenesis, resulting in suppression of pulmonary breast tumor metastases and increased life-span of tumor bearing, syngeneic BALB/c mice in a prophylactic setting.

Materials and methods

Animals, bacterial strains, and cell lines. Female BALB/c mice, 6 to 8 weeks of age, were purchased from the Scripps Research Institutes (La Jolla, CA) Rodent Breeding Facility. All animal experiments were performed according to the NIH Guides for the Care and Use of Laboratory Animals.

The double-attenuated *Salmonella typhimurium* strain RE88 (*dam*$^-$; *AroA*$^-$) was obtained from Remedyne Inc. (Santa Babara, CA).

Murine D2F2 breast cancer cell line was kindly provided by Dr. W-Z. Wei (Karmanos Cancer Institute, Detroit, MI) and cultured as previously described [49]. The murine high endothelial venule cell line (HEVc) was a gift from Dr. J.M. Cook-Mills (University of Cincinnati, Ohio). The mEndo$^+$-D2F2, were obtained by transfecting vector encoding full-length endoglin into D2F2 cells with DMRIE-C Reagent (Invitrogen, Carlsbad, CA). Endoglin positive cells were purified by FACSsort and maintained in selection medium containing Neomycin (1mg/ml).

Reverse transcription-PCR (RT-PCR)*:* Total RNA was extracted with the RNeasy mini kit or RNeasy tissue kit (Qiagen, Valencia, CA) from D2F2, HEVc cells or normal mouse spleen or liver. Reverse transcription was performed with 1μg of total RNA followed by PCR with specific endoglin primers: TCG ATA GCA GCA CTG GAT (forward), and ATC TAG CTG GAC TGT GAC (reverse). Primers for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used as control.

Immunohistochemistry: Frozen sections were fixed and stained with anti-endoglin Ab (BD PharMingen, San Diego, CA), followed by treatment with biotinylated anti-rat IgG Ab

and HRP-conjugated streptavidin (Vector Laboratories, Inc, Burlingame, CA). The DAB substrate (Sigma, St. Louis, MO) was added and slides were examined microscopically.

Vector construction and Western blotting. Full-length murine endoglin was cloned from tumor tissue. The vector was constructed based on the pCMV vector (Invitrogen). The empty vector served as a control. Western blot analysis was performed with transiently transfected COS-7 cell lysates using monoclonal rat anti-mouse endoglin Ab (Cymbus Biotechnology, UK).

Transformation of attenuated *S. typhimurium* and expression of endoglin *in vivo*. Attenuated *S. typhimurium* (*dam*$^-$*; AroA*$^-$) were transformed with DNA vaccine plasmids by electroporation [26]. Freshly prepared bacteria (1×10^8) were mixed with plasmid DNA (2μg) on ice in a 0.2-cm cuvette and electroporated at 2.5kV, 25μF, and 200Ω. Resistant colonies harboring the vaccine vectors were cultured and stored at -70°C after confirmation of their coding sequence.

Peyer's Patches (PPs) were dissected from the mouse small intestines [22] 24h after vaccination. Frozen sections were fixed, blocked and stained with unlabeled anti-endoglin Ab and Alexa 568-conjugated goat-anti-rat Ab (Molecular Probes), followed by biotin labeled anti-CD11c Ab and streptavidin-Alex 488 (Molecular Probe). Slides were air dried and mounted with Vectashield (Vector Laboratories) and analyzed by confocal microscopy with a Zeiss Axioplan/BioRad MRC 1024 confocal microscope.

Oral immunization and tumor challenge. BALB/c mice were divided into three experimental groups (n=8) and immunized 3 times at 1 wk intervals by gavage with 100 μl 5% sodium bicarbonate containing approximately 1×10^8 double attenuated *S. typhimurium*

harboring either empty vector (CMV) or murine endoglin vector (mEndoglin). All mice were challenged by i.v. injection of 1.5×10^5 D2F2 murine breast carcinoma cells 1 wk after the last immunization. Mice were monitored and sacrificed as indicated.

***In vivo* depletion of $CD4^+$ or $CD8^+$ T cell populations.** mAbs against CD8 (2.43: rat mAb, IgG_{2b}) or CD4 (GK1.5: rat mAb, IgG_{2b}) were purchased from The National Cell Culture Center (NCCC, Minneapolis, MN). Immunized mice were injected i.v. with anti-CD4 or anti-CD8 mAb (0.5 mg/mouse) 1 day before D2F2 tumor cell challenge, followed by weekly i.p. injection of mAbs until sacrifice.

***In vitro* cytotoxicity assay.** Cytotoxicity was performed by a standard [^{35}S] release assay [24;25]. Splenocytes were prepared from immunized mice 14 d after tumor cell challenge, and re-stimulated *in vitro* for 4 d on a monolayer of irradiated (1,000Gy) and mitomycin C-treated (80μg/10^7 cells, 45 min at 37°C) $mEndo^+$-D2F2 cells. Viable lymphocytes were separated by Lympholyte-M (Cenderlane, Ontario, Canada) gradient centrifugation and mixed at different ratios with {^{35}S} methionine-labeled target cells for 5h. Supernatants (100μl) were harvested and measured in a mixture with scintillation fluid. Percent specific lysis was calculated by the formula; $[(E-S)/(T-S)]\times100$, where E is the average experimental release, S the average spontaneous release, and T the average total release.

Flow cytometric analysis. T cell activation was assessed by staining freshly isolated splenocytes from vaccinated mice with FITC-labeled anti-CD8 Ab in combination with PE-conjugated anti-CD28 Ab. DCs were analyzed by PE-conjugated anti-CD80/CD86 mAbs in combination with FITC-labeled anti-CD11c mAbs. All reagents were obtained from BD Pharmingen (San Diego, CA). D2F2 or HEVc cells were stained with PE-labeled rat anti-

mouse endoglin mAb or isotype control Ab (both from Santa Cruz Biotechnology, Santa Cruz, CA). Flow cytometry were performed with a FACScan (Becton Dickinson, San Jose, CA) and the data analyzed with FlowJo software (Tree Star, Inc, Stanford, CA).

ELISPOT assay. The number of IFN-γ secreting cells was determined with an ELISPOT kit (BD Pharmingen) according to the manufacturer's instructions. Briefly, splenocytes were collected 10 d after the last immunization from all experimental groups. T cells were isolated from splenocytes on a Nylon Wool Column (Polysciences, Inc., Warrington, PA). Purified T cells (2×10^5/well) were cultured for 24 h with 2×10^4/well of irradiated (1,000Gy) D2F2 cells, mEndo$^+$-D2F2 cells or HEVc cells.

Evaluation of anti-angiogenic. One wk after the last vaccination, mice were injected s.c. near the abdominal midline with 500 μl of growth factor reduced Matrigel (BD Pharmingen) containing 400ng/ml bFGF (PeproTech, Princeton, NJ). Mice were injected 6 d later with 200μl (0.1mg/ml) isolectin B4 conjugated with fluorescein (Vector Laboratories) to stain the endothelium. Mice were sacrificed 15 min thereafter and Matrigel plugs were homogenized with RIPA lysis buffer (PBS, 1%NP-40, 0.5% sodium deoxycholate, 0.1% SDS). After centrifugation, the fluorescein content in the supernatant was quantified by fluorimetry at 490nm. Background fluorescence found in the non-injected control was subtracted in each case [1;27].

Statistical Analysis. The statistical significance of different finding between experimental groups and controls was determined by Student's t-test and considered significance it two-tailed P values was < 0.05.

Results

Determination of endoglin expression *in vitro* and *in vivo.*

Endoglin expression by the murine breast tumor cell line D2F2, the murine endothelial cell line HEVc and normal mouse spleen and liver was assessed by RT-PCR. Results indicated that HEVc strongly express endoglin (Fig. 1A). However, endoglin is absent in D2F2 cells or normal liver tissue under these experimental conditions. Low levels of endoglin expression in spleen were also observed (Fig. 1A). We further confirmed this finding by FACS analysis: HEVc cells express endoglin on the surface, but endoglin is not detectable on D2F2 tumor cells (Fig. 1B). However, endothelial cells in metastatic D2F2 lung tumor tissue highly express endoglin, while endoglin expression is barely detectable in normal lungs (Fig. 1C). Thus, these results confirm that the expression level of endoglin is significantly up-regulated on proliferating endothelial cells, despite the fact that D2F2 breast tumor cells themselves do not express detectable levels of endoglin [17].

The mEndoglin vaccine is delivered to Peyer's Patches.

To test our hypothesis that an oral DNA vaccine encoding endoglin induces a T cell-mediated immune response, we first inserted the entire gene encoding murine endoglin into the pCMV/myc/cyto expression vector (Fig. 2A). Protein expression of endoglin was demonstrated by a single band of expected molecular weight (90 Kda) detected by Western blots of lysates of COS-7 cells transiently transfected with mEndoglin (Fig. 2A).

Our oral DNA vaccination strategy using double attenuated *S. typhimurium* (*dam*$^-$; *AroA*$^-$) is designed to achieve successful *in vivo* delivery of plasmids to secondary lymphoid organs, i.e. PPs, to facilitate subsequent priming of specific T cells. To confirm endoglin expression after

vaccination, mice were sacrificed 24h after oral vaccine administration and PP collected from the thoroughly washed small intestine. Confocal microscopy demonstrated that a $CD11c^+$ DC sub-populations expressed endoglin intracellularly in PP of mEndoglin-vaccinated mice (Fig. 2B). However, endoglin was not detected in $CD11c^+$ DC cells from PPs of control mice.

The mEndoglin vaccine induces suppression of D2F2 breast tumor metastases.

We tested the efficacy of mEndoglin vaccine in a prophylactic setting, in which disseminated pulmonary metastases were induced in mice challenged by i.v. injection of 1.5×10^5 D2F2 breast carcinoma cells 1 week after the last vaccination. Whenever control mice showed signs of morbidity, all animals were sacrificed and evaluated for lung metastases and lung weights. Results (Fig. 3A) indicated that all mice receiving either PBS or empty vector presented with extensive disseminated pulmonary metastases. In contrast, all mEndoglin-vaccinated mice exhibited significant suppression of pulmonary metastases when compared to control mice ($P<0.05$). In addition, in survival studies, all control mice (PBS or CMV groups) died within 4 wk after tumor cell challenge due to extensive metastases; however, mice immunized with the mEndoglin vaccine had a 60% prolongation in life span (Fig. 3B).

The anti-tumor effects induced by the mEndoglin vaccine are mediated by $CD8^+$ T cells.

To determine the roles of cell subpopulations played in mEndoglin vaccine-induced suppression of pulmonary metastases, *in vivo* depletions of $CD4^+$ or $CD8^+$ were performed (Fig. 4). We observed that non-depleted, vaccinated mice effectively suppressed D2F2 pulmonary metastases when compared to the empty control vector mice ($p<0.05$); however, this suppression of pulmonary metastases was abrogated in mice depleted of $CD8^+$ T cell (Fig. 4), indicating that $CD8^+$ T cells play a major role in suppressing D2F2 pulmonary metastases. In contrast, *in vivo*

depletion of $CD4^+$ T cells did not significantly affect suppression of D2F2 pulmonary metastases, suggesting that $CD4^+$ T cells do not play a major role in anti-tumor effects induced by the mEndoglin vaccine.

The mEndoglin vaccine induces T cell and DC activation

We then investigated whether the anti-tumor activity of the mEndoglin vaccine correlated with T cell activation. This was evident from the increased expression of CD28, an important marker of activated T cells (Fig. 5), especially since optimal T cell activation is critically dependent on the ligation of CD28 with co-stimulatory molecules CD86 and CD80 on DCs. In this regard, FACS analyses of splenocytes obtained from vaccinated mice clearly demonstrated that the expression of both CD80 and CD86 on $CD11c^+$ DCs was up-regulated when compared with control animals (Fig. 5).

Immunization with the mEndoglin vaccine evokes endoglin-specific CTLs

In order to assess whether $CD8^+$ T cells are able to specifically lyse endoglin-positive target cells, we generated endoglin-expressing D2F2 cells ($mEndo^+$-D2F2) by transfection of D2F2 cells with the endoglin plasmid. These cells expressed endoglin on the surface (Fig. 6A), in comparison to wild-type D2F2 cells that did not express endoglin (Fig 1B).

ELISPOT analysis for IFNγ secretion was performed to determine the frequency of endoglin-specific T cells in mEndoglin-vaccinated mice. The number of spots markedly increased when such cells were co-incubated with irradiated $mEndo^+$-D2F2 cells as stimulator when compared to stimulation with wild-type D2F2 cells (Fig. 6B). These data indicate the success in expanding endoglin-specific cells in mEndoglin vaccinated mice.

Furthermore, we determined whether such activated T cells could eliminate endoglin-expressing endothelial target cells. The results (Fig. 6C) indicate that endothelial HEVc target cells, which naturally express endoglin, are susceptible to lysis by effector cells obtained from mEndoglin-vaccinated. In contrast, T cells from control mice showed low level of killing (Fig. 6C).

We next examined the specificity of the vaccine-induced cytotoxicity. In fact, mEndo^{+}-D2F2 target cells were 2 times more sensitive to CTL killing than wild-type D2F2 cells ($p<0.05$, Fig. 6D). Moreover, mEndo^{+}-D2F2 cells were more susceptible to lysis by effector cells obtained from mEndoglin vaccinated mice than by those from control mice (Fig. 6D). These data indicate that the mEndoglin vaccine effectively induced the specific elimination of endoglin-positive target cells.

The mEndoglin vaccine elicits suppression of angiogenesis

We assessed whether the mEndoglin vaccine could suppress angiogenesis. In this regard, a Matrigel assay revealed a significant decrease in neovascularization only in mice immunized with mEndoglin vaccine (Fig. 7). In fact, quantification of relative fluorescence intensity, measured after *in vivo* staining of mouse endothelium with FITC-conjugated lectin, clearly indicated that the angiogenic process in such experimental animals decreased significantly in comparison to control mice ($P<0.05$).

Discussion

An oral DNA vaccine encoding murine endoglin, which is primarily over-expressed by proliferating endothelial cells in the angiogenic tumor vasculature, effectively induced an endoglin-specific $CD8^+$ T cell-mediated immune response. This immune response broke peripheral tolerance against the endoglin self-antigen and presumably suppressed tumor angiogenesis, resulting in the suppression of pulmonary D2F2 breast carcinoma metastases in a prophylactic setting.

The rationale for using double attenuated *S. typhimurium* as a vaccine carrier is based on our prior data [30;32;33;48], including the finding that transformation of such bacteria with a DNA plasmid encoding a tumor antigen and their subsequent oral administration by gavage leads to delivery of the vaccine via the small intestine and M cells into PPs. There, the attenuated bacteria are phagocytosed, primarily by DCs in the subepithelial dome of this secondary lymphoid organ [23], and then die due to their mutations and liberate the DNA. This is followed by transcription and translation of the DNA and processing of proteins or peptides in the proteosomes of these APCs, ultimately leading to the formation of antigen peptide/MHC class I Ag complexes in the cytosol which are delivered to the cell surface and presented to T cell receptors. In this regard, intralymphatic immunization with naked DNA was reported to be most effective since it is 100- to 1,000- fold more efficient inducing strong and biologically relevant $CD8^+$ CTL responses over traditional i.m., s.c., or i.v. routes of immunization [28]. Consequently, vaccination with naked DNA appears to be optimal when targeted to secondary lymphoid organs such as PP. In addition, in this draining lymph node, effective cross-priming of $CD8^+$ cells may also possibly be achieved without $CD4^+$ T cell help [50].

Antiangiogenic therapies generally take two approaches: 1) targeting preexisting blood vessels or 2) preventing the development of the tumor neovasculature. Since our vaccination was performed in a prophylactic setting, where vaccination preceded tumor cell challenge, $CD8^+$ T cell responses induced by our mEndoglin vaccine likely interfered with the development of the tumor angiogenic blood vessels which, in turn, prevented the establishment of D2F2 pulmonary metastases.

The well-established, high level expression of endoglin by proliferating endothelial cells of both peri- and intratumoral blood vessels [6;15;39] makes endoglin an excellent target for antiangiogenic therapy, particularly in attempts to prevent the development of tumor blood vessels. Since in our experimental system, endoglin is only over-expressed by proliferating endothelial cells in angiogenic blood vessels (Fig. 1), targeting proliferating endothelial cells has several additional advantages over targeting tumor cells. These include the following: first, the avoidance of tumor antigen heterogeneity and the down-regulation of MHC class I antigens, both of which seriously limit effective T cell-mediated immune responses against tumor cells; second, the specific targeting of the antiangiogenic intervention to proliferating endothelial cells in the tumor neovasculature limits its toxicity; third, the direct contact of the vasculature with the circulation makes for efficient access of therapeutic agents since the target tissue can be reached unimpaired by anatomical barriers such as the blood-brain barrier or encapsulated tumor tissue [2;9;13;37]; fourth, since the therapeutic target is tumor-independent, killing of proliferating endothelial cells in the tumor microenvironment could be effective against a variety of solid tumors [8;11;21;35;36].

Taken together, our data indicate that the endoglin-based DNA vaccine, delivered to PP in the small intestine by double attenuated *S. typhimurium,* evoked an effective $CD8^+$ T cell-mediated anti-tumor immune response. Importantly, this response was shown to be specifically directed against endoglin expressed by proliferating endothelial cells, and presumably resulted in the suppression of angiogenesis in the breast tumor neovasculature. This included the ability of T cells from mEndoglin-vaccinated mice to specifically lyse both $mEndo^+$-D2F2 and endothelial target cells, the latter naturally express endoglin. The up-regulation of the T cell activation marker, CD28, and of co-stimulatory molecules CD80/CD86 on DCs provided further evidence for the activation of these cells. This type of activation is presumably of key importance to achieve a T cell-mediated immune response leading to the limitation of angiogenesis in the tumor vasculature, as well as to the suppression of breast tumor growth and pulmonary metastases in a prophylactic tumor model.

In conclusion, we anticipate that this novel, oral DNA vaccine targeting endoglin might ultimately lead to a successful clinical application aiding in the prevention and therapy of human breast cancer.

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Figures legends:

Fig. 1. Expression of murine endoglin by tumor cells lines and normal mouse tissues. **(A)** RT-PCR analysis of endoglin expression by wild-type D2F2 breast carcinoma cells, HEVc endothelial cells, as well as normal mouse spleen and liver. Total RNA extraction and RT-PCR were performed according to "*Material and Methods*" and GAPDH was used as control for total RNA loading. **(B)** FACS analyses of endoglin expression on the cell surface of D2F2 cells and HEVc cells, both stained with PE-conjugated anti-endoglin Ab. PE-labeled Rat IgG Ab served as isotype control (black line). **(C)** Comparison of endoglin expression levels on normal lungs and lung metastases of D2F2 tumor induced by i.v. injection of breast tumor cells (1×10^6 cells/mouse). Mice were sacrificed when inoculated animals were moribund and frozen lung sections analyzed by immunostaining with anti-endoglin Ab (×20 magnification).

Fig. 2. Schematic of a vector encoding murine endoglin and expression *in vitro* and *in vivo*. **(A)** Protein expression of endoglin was detected by Western blotting of endoglin-transfected COS-7 cell lysates with anti-endoglin Ab. **(B)** Endoglin expression of $CD11c^+$ DCs in Peyer's patches isolated 24 h after vaccination. Sections were stained with Ab against CD11c (green) and Ab against endoglin (red), and examined for expression by confocal microscopy. Samples derived from mice administered with empty vector served as a negative control.

Fig. 3. Suppression of pulmonary metastases of D2F2 breast carcinoma by the mEndoglin vaccine in prophylactic tumor models. Lung metastases were induced by i.v. injection of 1.5×10^5 D2F2 cells, 1 week after the last immunization. Experiments were terminated 27 d

after tumor cell inoculation and the extent of pulmonary tumor metastases evaluated based on lung weights. **(A)** Lung weights are depicted following immunization with PBS, empty vector or mEndoglin vaccine. Symbols within bar graphs of experimental groups each represent a single mouse (n=8). Normal lung weight is approximately 200mg. * $p<0.05$ when compared with either PBS or empty vector. **(B)** Kaplan-Meier survival plots of different groups of mice (n=8). Differences in survival times between the mEndoglin group and control groups were statistically significant according to Kaplan-Meier analyses ($p<0.05$). Experiments were repeated three times with similar results.

Fig. 4. Increased incidence of pulmonary D2F2 metastases of vaccinated mice that were depleted of $CD8^+$ T cell populations. Mice were immunized orally 3 times at weekly intervals with either DNA encoding endoglin or the empty vector. Anti-CD8 mAb (2.43:mouse mAb, IgG2b) or anti-CD4 mAb (GK1.2: rat mAb, rat IgG2b), were each injected i.v. 7d after the last immunization. The following day, experimental lung metastases were induced in a prophylactic setting by i.v. injection of 1.5×10^5 D2F2 cells followed by additional weekly i.p. injection of anti-CD8 or anti-CD4 mAb to maintain the depleted state of subset T cells until the termination of the experiments. Mice were sacrificed 27 d after D2F2 tumor cell challenge and lung weights established to determine the extent of pulmonary metastases. The data shown in bar-graphs reveal the average ± SD of lung weights in each experimental group (n=8). Statistically significant differences in lung weights between groups of mice treated with either the empty vector and non-depleted as well as $CD4^+$ and $CD8^+$ depleted T cells are shown. The data are representative of two independent experiments.

Fig. 5. The mEndoglin vaccine induced activation of T cell and DCs. Two-color flow cytometric analyses are performed with splenocytes from vaccinated mice 1 week after tumor cell challenge. For T cell activation, PE-conjugated anti-CD28 mAbs were used in combination with FITC-conjugated anti-mouse CD8 mAb (upper panel). For DC analyses, splenocytes were stained with FITC-labeled anti-CD11c mAb in combination with PE-conjugated anti-CD80 (middle panel) or anti-CD86 mAbs (lower panel). Y-axis represents % of double positive cells as to the total $CD8^+$ or total $CD11c^+$ cells (mean ± SD, n=4). Differences between the control groups and the treatment group were statistically significant ($P<0.05$).

Fig. 6. Vaccination against endoglin induces specific T-cell responses. **(A)** FACS analyses of endoglin expression on endoglin-transfected D2F2 tumor cells ($mEndo^+$-D2F2). Cells were stained with PE conjugated anti-endoglin Ab. Rat IgG Ab was used as an isotype control (black line). **(B)** ELISPOT analyses of IFNγ producing cells using different stimulator cells. Splenocytes, enriched for $CD8^+$ cells, were isolated from vaccinated mice and incubated for 24h with either irradiated wild-type D2F2 cells, $mEndo^+$-D2F2, or HEVc endothelial cells. The mean spot number of each group is shown (n=3, mean ± SD). **(C)** T cell-specific cytotoxicity against endoglin-positive HEVc endothelial target cells. Splenocytes were isolated from vaccinated mice 10 d after tumor cell challenge. A [^{35}S]-release assay was performed at different effector-to-target cell ratios with splenocytes being re-stimulated with irradiated $mEndo^+$-D2F2 cells for 5 d and [^{35}S] methionine labeled HEVc used as target cells. The data depict average ± SD of triplicate wells. Similar results

were obtained in three independent experiments. **(D)** Sensitivity of mEndo^{+}-D2F2 and wild-type D2F2 cells (mEndo^{-}-D2F2) to CTL killing. [^{35}S] methionine labeled wild-type D2F2 or mEndo^{+} D2F2 target cells were co-incubated with effectors at E/T =1:12.5. Similar results were obtained in three independent experiments. ∗, $P<0.05$ compared with control wild-type D2F2 target cells.

Fig. 7. Suppression of angiogenesis by the mEndoglin vaccine. One week after the last vaccination, Matrigel was implanted s.c. into the midline of the abdomen of either control mice (n=7) or vaccine-treated mice (n=8), and vessel growth quantified by staining of endothelium with FITC-labeled Isolectin B4 as described in *Material and Methods*. The average fluorescence of extracts is measured by fluorimetry at 490nm and depicted by bar graphs (mean ± SD; $P<0.05$).

Fig. 1.

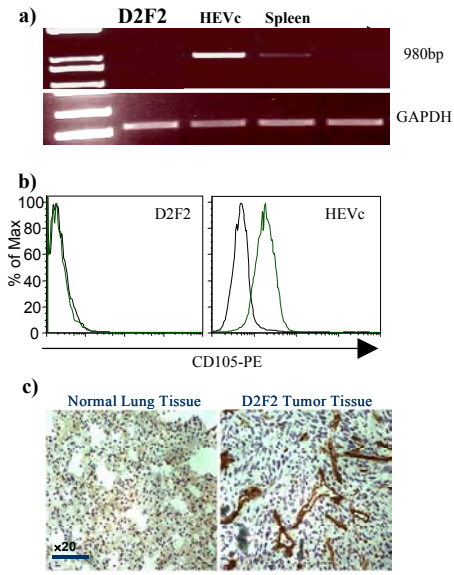
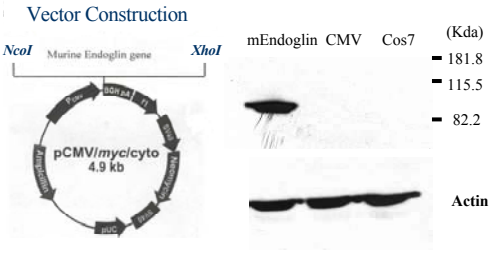

a)
D2F2
HEVc
Spleen
980bp
GAPDH
b)
D2F2
HEVc
% of Max
100
80
60
40
20
0
CD105-PE
c)
Normal Lung Tissue
D2F2 Tumor Tissue
x20

Fig. 2.

a)


Vector Construction
NcoI
Murine Endoglin gene
XhoI
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4.9 kb
mEndoglin CMV Cos7
(Kda)
181.8
115.5
82.2
Actin

b)

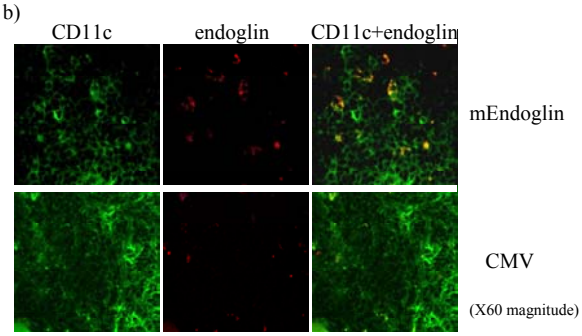

CD11c
endoglin
CD11c+endoglin
mEndoglin
CMV
(X60 magnitude)

Fig. 3.

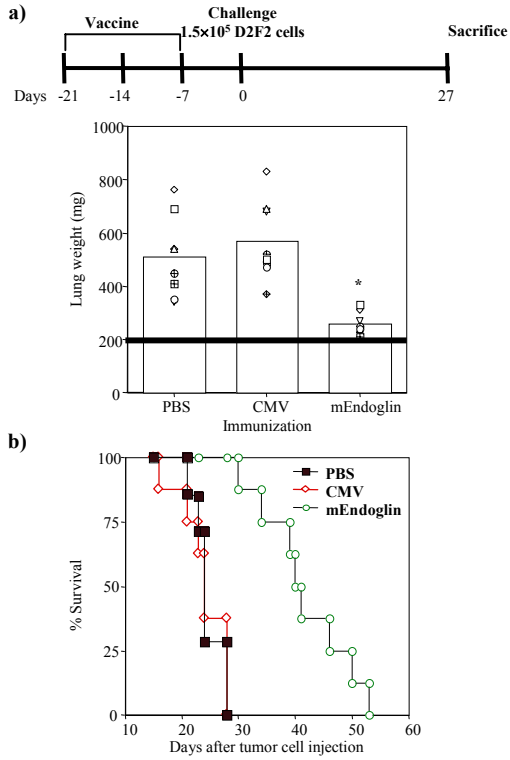

a)
Vaccine
Challenge
1.5×10^5 D2F2 cells
Sacrifice
Days -21 -14 -7 0 27
Lung weight (mg)
1000
800
600
400
200
0
PBS
CMV
mEndoglin
Immunization
b)
% Survival
100
75
50
25
0
10 20 30 40 50 60
Days after tumor cell injection
PBS
CMV
mEndoglin

Fig. 4.

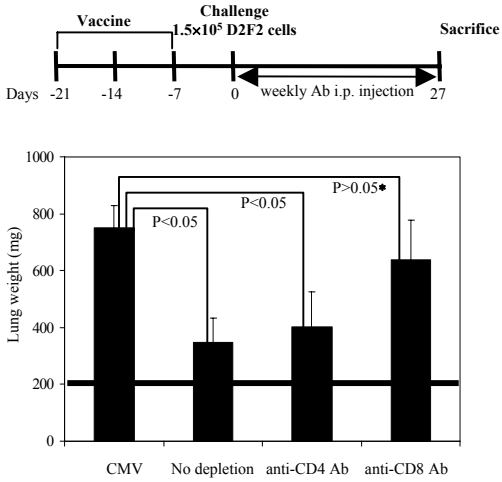

Vaccine
Challenge
1.5×10^5 D2F2 cells
Sacrifice
Days -21 -14 -7 0 weekly Ab i.p. injection 27
Lung weight (mg)
1000
800
600
400
200
0
P<0.05
P<0.05
P>0.05*
CMV
No depletion
anti-CD4 Ab
anti-CD8 Ab

Fig. 5.

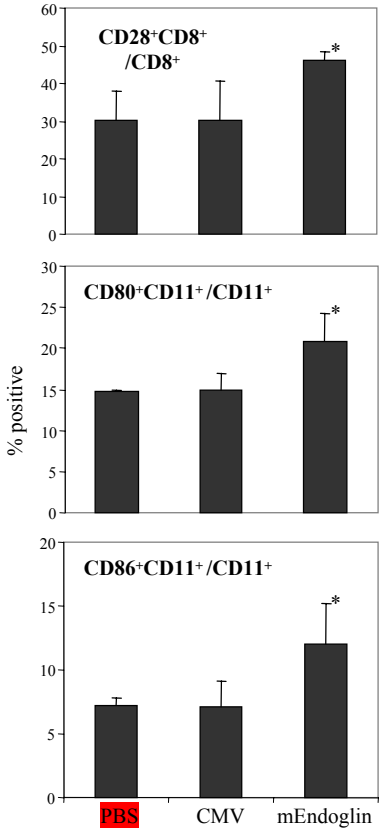

CD28+CD8+ /CD8+
CD80+CD11+/CD11+
CD86+CD11+/CD11+
% positive
PBS
CMV
mEndoglin

Fig. 6.

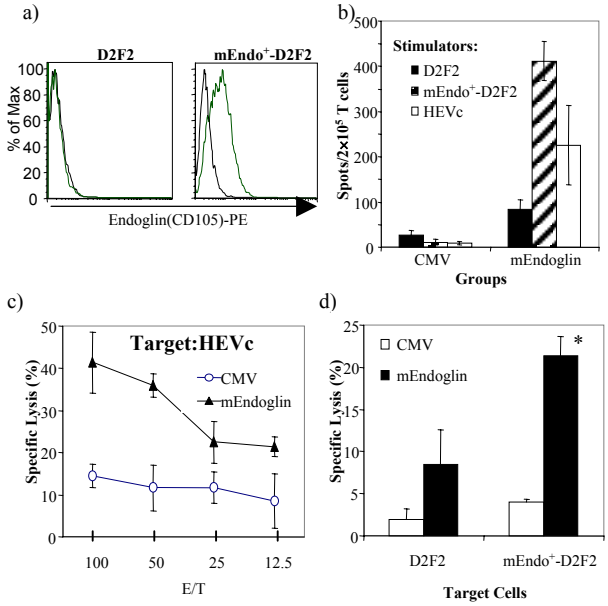
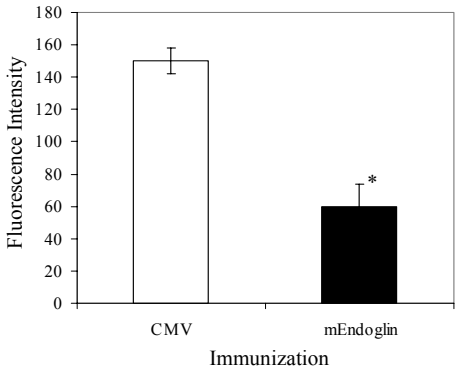

a)
D2F2
mEndo+-D2F2
% of Max
Endoglin(CD105)-PE
b)
Stimulators:
D2F2
mEndo+-D2F2
HEVc
Spots/2x10^5 T cells
CMV
mEndoglin
Groups
c)
Target:HEVc
CMV
mEndoglin
Specific Lysis (%)
E/T
d)
CMV
mEndoglin
Specific Lysis (%)
D2F2
mEndo+-D2F2
Target Cells

Fig. 7.


180
160
140
120
100
80
60
40
20
0
Fluorescence Intensity
*
CMV
mEndoglin
Immunization

Targeting Tumor-Associated Macrophages: A Novel Strategy against Breast Cancer

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Running Title: Targeting tumor-associated macrophages against breast cancer

Key Word: Tumor-associated macrophages, Legumain, breast cancer stroma

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Abstract

Tumor-associated macrophages (TAMs) are associated with tumor progression and metastasis. Here, we demonstrate for the first time that Legumain, a member of the asparaginyl endopeptidase family functioning as a stress protein, overexpressed by TAMs, provides an ideal target molecule. In fact, a Legumain-based DNA vaccine served as a tool to prove this point as it induced a robust $CD8^+$ T cell response against TAMs, which dramatically reduced their density in tumor tissues and resulted in a marked decrease in pro-angiogenesis factors released by TAMs such as TGF-β, TNF-α and VEGF. This, in turn, led to a decisive suppression of both, tumor angiogenesis and tumor growth and metastasis. Importantly, the success of this strategy was demonstrated in murine models of metastatic breast, colon and non-small cell lung cancers where 72% of vaccinated mice survived lethal tumor cell challenges and 62% were completely free of metastases. In conclusion, attenuation of TAMs in the tumor stroma effectively altered the tumor microenvironment involved in tumor angiogenesis and progression to markedly suppress tumor growth and metastasis. Gaining better insights into the mechanisms required for an effective intervention in tumor growth and metastasis may ultimately provide new therapeutic targets and better anticancer strategies.

Introduction

A novel anti-tumor strategy is to immunize against molecules overexpressed by TAMs and thereby remodel the tumor microenvironment which attracts these macrophages and mediates their function. [1,2]. TAMs consist primarily of a polarized M2 ($CD206^+$, $F4/80^+$) macrophage population with little cytotoxicity for tumor cells because of theirlimited

production of nitric oxide and proinflammatory cytokines [3]. TAMs also possess poor antigen presenting capability and effectively suppress T cell activation. In fact, these macrophages of M2 phenotype actually promote tumor cell proliferation and metastasis by secreting a wide range of growth and pro-angiogenesis factors as well as metalloproteinases, and by their involvement in signaling circuits that regulate the function of fibroblasts in the tumor stroma [4]. Currently, anti-TAM effects induced by small molecule inhibitors contributed to tumor suppression as reported by several research groups [5,6]. For example, the antineoplastic agent Yondelis has a selective cytotoxic effect on TAMs, thereby significantly reducing their production of IL6 and CCL2 which, in turn, contribute to growth suppression of inflammation-associated human tumors [7]. Another such example is provided by a biphosphonate compound, Zoledronic acid, which suppresses MMP9 secretion by TAMs, thereby inhibiting tumor metalloproteinase activity and diminishing the association of VEGF with its tyrosine kinase receptors on proliferating endothelial cells [8]. In a different experimental model, the chemokine CCL5 was shown to be key in the recruitment of TAMs and an antagonist of this chemokine reduced the tumor infiltrate and slowed tumor growth [9].Hence, although the therapeutic targeting of TAMs is still in its infancy, initial clinical results are encouraging as they suggest that targeting TAMs may complement more conventional cancer treatment regimens.

The selection of Legumain as a target for tumor therapy is based on the fact that the gene encoding this asparaginyl endopeptidase was found to be highly up-regulated in many murine and human tumor tissues [1,10,11], but absent or only present at very low levels in all normal tissues from which such tumors arise. In this regard, we recently discovered that

Legumain is heavily overexpressed by TAMs in murine breast tumor tissues by using gene expression profiling and immunohistochemistry. Importantly for our studies, TAMs have a particularly abundant expression in the tumor stroma[10] and express high levels of Legumain in this tumor microenvironment. In contrast, classical macrophages of M1 phenotype, that perform key immune-surveillance and antigen presentation functions, do not express Legumain. Consequently, targeting TAMs that overexpress Legumain does not interfere with the biological functions of (M1) macrophages, including cytotoxicity and antigen presentation [12-14].

Based on these findings, we hypothesized that targeting TAMs which overexpress Legumain will reduce their density and thereby remodel the tumor microenvironment. This should lead to the down-regulation of a wide variety of tumor growth factors, pro-angiogenesis factors and metalloproteinases released by these M2 macrophages and thereby decisively suppress angiogenesis of tumors as well as their growth and metastasis. To test our hypothesis, a legumain-based DNA vaccine served as a tool to eliminate TAMs in murine models of colon, breast and lung tumor metastases.

Results

1. Legumain serves as a target to kill TAMs overexpressed during tumor progression: It is well known that TAMs play a key role in tumor progression and metastasis[5]. Therefore, targeting of these M2 macrophages represents a novel anti-tumor strategy. We initially identified Legumain as a significant marker molecule of TAMs since it was highly overexpressed on these cells in the tumor microenvironment and stroma. To this end, we isolated TAMs from murine 4T1 breast tumor tissue and

demonstrated by Flow cytometry that Legumain was highly overexpressed on CD206 and F4/80 double positive M2 macrophages, especially when compared to normal M1macrophages in the spleen (Fig. 1a). This result was also confirmed by immunohistochemical analyses indicating that TAMs could be visualized by H/E staining (Fig. 1b top panel). Legumain overexpression was further indicated by double staining with anti-Legumain Ab (green) combined with anti-CD68 Ab (red) (Fig.1b bottom panel). These data demonstrate that infiltrating TAMs are a disproportionally large cell subpopulation in 4T1 tumor tissue and that Legumain is a potentially effective target for killing TAMs.

2. Induction of Legumain expression on TAMs by Th2 cytokines: In order to determine whether Legumain expression on TAMs was induced by such Th2 cytokines as IL-4, IL-10 and IL-13, we made use of a murine macrophage cell line, RAW, co-cultured with these cytokines. This resulted in a significant increase in $CD206^{+}$, $F4/80^{+}$ expression by these RAW cells, concurrent with an upregulation of Legumain (Fig. 1c). These results were confirmed by Western blotting (Fig. 1d). However, we found no evidence for Legumain expression by tumor cell lines when cultured with these same cytokines (data not shown). These findings suggest that Th2 cytokines such as IL-4, IL-10 and IL-13, released by tumor and tumor stromal cells and accumulated in the tumor microenvironment, could potentially induce the proliferation and transformation from M1 macrophages to a population with M2 phenotype which overexpresses Legumain.

3. Targeting of TAMs suppresses tumor metastases: Growth and proliferation of tumor metastases are highly coordinated with the presence of TAMs, and therefore

targeting of this macrophage subpopulation leads to a suppression in tumor growth and metastases. To test this hypothesis, we generated an expression vector for a DNA vaccine encoding Legumain. Fig. 2a schematically depicts the vector construction map based on the pCMV/myc/cyto vector backbone. The gene encoding Legumain was fused to the C-terminal of mutant polyubiquitin (pUb-Legumain) and the entire fragment was then inserted between the PstI and NotI restriction sites while protein expression was demonstrated by Western blotting. We further tested our hypothesis that reducing the number of TAMs by our Legumain-based DNA vaccine can effectively inhibit spontaneous 4T1 breast cancer metastases or experimental metastases of either D121 non-small cell lung or CT26 colon carcinomas. Thus, in a prophylactic setting, C57BL/6J mice were immunized three times with either PBS, empty vector or pLegumain carried by attenuated *Salmonella typhimurium*. One week after the last immunization, these mice were challenged i.v. with 2×10^5 D121 non-small lung tumor cells and 24 d thereafter experimental lung metastases were measured and analyzed (Fig. 2b). In the two control groups, the average lung weight was significantly greater than that of the treatment group (Fig. 2 b). Similar results were obtained in the CT26 colon tumor model in syngeneic BALB/c mice (Fig. 2b).

In a more demanding therapeutic setting, BALB/c mice were first challenged with 4T1 breast cancer cells and then immunized with the Legumain-based DNA vaccine. Twelve days after challenge with 4T1 tumor cells, the primary tumor was surgically resected and the resulting life span curve indicated that 75% (6/8) of the mice immunized with pLegumain survived for 3 months (Fig. 2c). In contrast, mice in the control groups all died within one month. The results of a further experiment confirmed these data as the

metastasis score and lung weights, measured 25 d after tumor cell challenge, decreased significantly when compared with the two control groups (Fig. 2d). These data indicate that the Legumain-based DNA vaccine effectively suppresses tumor cell growth and metastases in both the 4T1 breast cancer and the D121 non-small cell lung cancer models. Combined with surgery, this vaccine could indeed significantly extend the life span of mice by inhibiting tumor cell metastases in these very challenging therapeutic mouse tumor models (Fig. 2c).

4. Targeting Legumain induces a specific $CD8^+$ CTL response decreasing the TAM population in the tumor stroma: Immunization against Legumain induced a specific T cell response against TAMs that highly express this asparaginyl endopeptidase. This was demonstrated by a ^{51}Cr release assay where splenocytes isolated from mice successfully immunized with this vaccine were effective in killing RAW macrophages which expressed high levels of Legumain after culture with cytokines IL-4, IL-10 and IL-13 (Fig. 3a; left panels); however, these same splenocytes failed to induce cytotoxic killing of cells which lacked Legumain expression (Fig. 3a right panels), indicating the specificity of this T cell response. The results depicted in Fig. 3b demonstrate a dramatic decrease in the $F4/80^+/CD206^+$ macrophage population after DNA vaccine treatment. These data were also confirmed by immunohistochemical staining (Fig. 3c).

5. MHC-class I restricted $CD8^+$ CTLs specifically kill TAMs: In gaining some insight into the immune mechanisms involved in the killing of TAMs we found that DCs in the Peyer's patches of successfully immunized mice were activated 3 d after vaccination with pLegumain as indicated by the upregulated DC activation marker, CD11c (Fig. 4a).

Furthermore, $CD8^+$ T cell activation was found to be specific for Legumain as indicated by double staining for INF-γ and CD8 on splenocytes obtained from successfully vaccinated mice, (Fig. 4b), and by the specific release of INF- γ by activated T cells stimulated with Legumain-positive cells (Fig. 4c). In addition, in vivo immune depletion of $CD4^+$ or $CD8^+$ T cells revealed that only $CD8^+$ T cells play a major role in the specific cytotoxic killing of TAMs since their depletion completely abrogated this killing effect (Fig. 4d). This specific cytotoxity was MHC-class I restricted because killing was specifically inhibited by anti-H-$2D^d$/H-$2K^d$ antibodies (Fig. 4d). Taken together, our results suggest that the Legumain-based DNA vaccine first activated DCs in Peyer's Patches, after which these cells presented Legumain peptides through the MHC-class I pathway to the TCR on activated $CD8^+$ T cells, resulting in a specific cytotoxic $CD8^+$ T cell response abrogating TAMs.

6. Abrogation of TAMs in the tumor stroma reduces the release of tumor growth factors, pro-angiogenesis factors and MMP-9: TAMs can influence tumor metastasis in several ways as they secrete a wide variety of tumor growth factors, pro-angiogenesis factors and tumor-associated enzymes that stimulate tumor angiogenesis and tumor growth and metastasis. In an effort to assess whether the elimination of TAMs actually reduced the release of some of these factors, serum and tumor tissue cells were collected from vaccinated mice. Freshly isolated tumor cells were cultured and their supernatants collected at 24 and 48 h respectively. An ELISA, performed to quantify TNF-α, VEGF and TGF-β, indicated a significant reduction in TNF-α and VEGF in both tumor cell supernatants and serum; however, TGF-β was reduced only in cell supernatants but not in

serum (Fig. 5a). Immunohistological staining confirmed a decrease in the expression of these factors (Fig. 5b). Importantly, there also was a marked anti-angiogenic effect after eliminating TAMs in the tumor stroma, particularly since these M2 macrophages produced a wide range of pro-angiogenesis factors. This was established by Matrigel assays that detected new blood vessel growth *in vivo,* an effect that could be quantified by staining the endothelium with FITC labeled isolectin B4. These results clearly show that vessel growth was significantly reduced after vaccination with pLegumain (Fig. 5c). In addition, a significantly decreased tumor cell migration was found when comparing treatment and control groups (Fig. 5d) in a migration and invasion assay, which indicated that these characteristics of tumor cells changed after the vaccine-induced remodeling of the tumor microenvironment caused by the reduction in TAMs.

Discussion

This study established the new paradigm that a reduction in the density of TAMs in the tumor stroma decreases the release of factors potentiating tumor growth and angiogenesis. This, in turn, remodels the tumor micro-environment such as to markedly suppress tumor cell proliferation, vascularization and metastasis. However, targeting TAMSs in the tumor stroma raises the concern that their abrogation could interfere with the normal immunological functions of these important components of the innate immune system. We addressed this question in view of the fact that circulating monocytes are versatile precursors with the ability to differentiate into the various forms of specialized macrophages [3]. In fact, the cytokine milieu profoundly affects the differentiation and function of tissue macrophages and their functional polarization has been defined [3,15,16].

Thus, macrophages activated by bacterial products and Th1 cytokines are regarded as M1, or classically activated macrophages with high bactericidal activity and cytotoxic function against tumor cells. However, macrophages activated by such Th2 cytokines as IL-4, and IL-13, or immunosuppressors such as vitamin D3 and IL-10 are defined as M2 macrophages with low cytotoxic functions but high tissue-remodeling activity. Whereas M1 cells have immunostimulatory properties and defend the host against pathogenic infections, M2 cells attenuate acute inflammatory reactions, potently scavenge cellular debris, and secrete a variety of pro-growth and angiogenesis factors essential for the repair of injured tissues. Indeed, a growing body of evidence indicates that TAMs are skewed toward M2 macrophages and produce a variety of pro-tumor growth and angiogenesis factors as well as immunosuppressive molecules [1,6,17,18]. Thus, the presence of TAMs at the tumor site and the continuous expression and release of their products may favor, rather than antagonize tumor progression and metastasis.

In our study we could demonstrate that TAMs express abundant levels of CD206, a mannose receptor, which is preferentially expressed on M2 macrophages[18,19]. We also established simultaneously that this population of macrophages expressed high levels of Legumain. Importantly, we found that Th2 cytokines IL-4, IL-10 and IL-13 could up-regulate the expression of CD206 and Legumain on the macrophage cell line RAW. This finding can best be understood when one considers that M1macrophages are derived from peripheral blood and proliferate into M2 macrophages once they are recruited into tumor sites where IL-4, IL-13 and IL-10 are released by tumor cells and tumor stromal cells [3,20-22,22]. Thus, targeting of M2 macrophages expressing Legumainnot only benefits

suppression of tumor growth and metastases but also maintains the normal functions of macrophages with M1 phenotype.

The relationship between infiltration by TAMs and prognosis in tumor patients has been investigated in several studies [1,5,23,24] which concluded that the greater the macrophage infiltration, the worse the prognosis. Several lines of evidence indicate that a symbiotic relationship exists in the tumor stroma between cancer cells and TAMs, whereby cancer cells attract TAMs and sustain their survival, while TAMs respond to tumor-derived molecules by producing important growth factors and extracellular matrix enzymes which, in turn, stimulate tumor proliferation, angiogenesis, and invasion of surrounding tissues [14,17,25,26]. Thus, the attenuation of TAMs in the tumor environment can serve as an effective strategy to remodel the tumor stroma and alter the tumor microenvironment [27]. In our study, a DNA construct encoding Legumain evoked a robust CTL response against this asparaginyl endopeptidase which functions as a stress protein that is highly overexpressed by TAMs. This immune response was shown to be MHC-I class I antigen-restricted and $CD8^+$ T cell specific. Importantly, our data demonstrate that after immunization with the Legumain-based DNA vaccines, the density of double positive $CD206^+$ and $F4/80^+$ macrophages; i.e. TAMs, decreased dramatically. Furthermore, a variety of factors such as VEGF, MMP-9 and TGF-β that are released by TAMs was shown to be at low levels in both the supernatant of cultured tumor cells and mouse serum. In this regard, it is well known that VEGF and metalloproteinase MMP-9 play important roles during the formation of the tumor vasculature and initiation of tumor angiogenesis. TAMs are important in this regard since they produce both VEGF and MMP-9 [28]. Progressively intensifying angiogenesis is associated with the upregulated

expression of VEGF [29] and extracellar proteases, such as MMP-9 [8,28], whereas TGF-β is known as an important growth factor involved in the migration of tumor cells towards blood vessels. In fact, TGF-β can provide proliferative and anti-apoptotic signals to tumor cells as well as activate urokinase-type plasminogen activators (uPA) that might contribute to the extracellular matrix breakdown which is required for vascular invasion to occur [30]. Significantly, our data demonstrate that once TAMs were abrogated in the tumor tissue by specific $CD8^+$ CTLs, the tumor cells changed their character by becoming less malignant and less invasive. Also, the formation of a neovasculature in tumor tissues was reduced since all of the factors released by TAMs that contribute to tumor angiogenesis were drastically reduced.

Furthermore, our contention that a therapeutic approach using a Legumain-based DNA vaccine to target TAMs holds much promise was supported by data obtained in three tumor metastasis models used in our study. Thus, in the 4T1 spontaneous mouse breast carcinoma metastasis model, a significant increase was obtained in a life span study where 75% (6 out of 8) mice survived up to 3 months after 4T1 tumor cell inoculation into the mammary gland, once surgical recision of the primary tumor was followed by treatment with the Legumain-based DNA vaccine. It was particularly impressive that 62% (5 out of 8) mice revealed no lung metastases at all. Similar results were obtained in the other two tumor models, i.e. D121–non-small cell lung carcinoma and CT-26 colon carcinoma. These additional confirmatory data strengthen our contention that targeting of TAMs in order to remodel the tumor microenvironment might be a universal anti-tumor strategy which suppresses tumor cell invasion and metastases by reducing the

concentration of factors released by TAMs that otherwise promote tumor growth and metastasis.

In summary, we critically evaluated the anti-tumor efficacy of targeting tumor-associated macrophages (TAMs) via the induction of a specific $CD8^+$ T cell response against Legumain which we identified for the first time as a highly expressed target molecule on TAMs. In these experiments, we demonstrated that abrogation of TAMs in tumor tissues effectively decreased those pro-tumor growth and angiogenesis factors released by TAMs. It is likely that such an anti-tumor strategy could be widely applicable and relevant for possible clinical applications.

Materials and Methods

Animals, Bacterial Strains and Cell Lines. Female BALB/c and C57BL/6 mice, 6-8 wk of age, were purchased from The Scripps Research Institute Rodent Breeding Facility. The double attenuated *S. typhimurium* strain RE88 (*aroA*$^-$*;dam*$^-$) was obtained from Remedyne Corporation (Santa Barbara, CA.). The murine CT-26 colon cancer cell line was kindly provided by Dr. I.J. Fidler (MD Anderson Cancer Center, Houston,TX.) and the murine D121 non-small cell lung carcinoma cells were a gift from Dr. L. Eisenbach (Weizmann Institute of Science, Rehovot, Israel). The murine 4T1 breast carcinoma cells were kindly provided by Dr. Suzanne Ostrand-Rosenberg (University of Maryland, Baltimore, MD.).

Western blots and Immunohistochemistry. *Western blots*: analyses were performed on the total protein from cell lysate homogenates, using a polyclonal primary rabbit anti-murine Legumain Ab as well as an anti-murine β-actin Ab as a loading control (Santa

Cruz Biotechnology Inc., Santa Cruz, CA.). Specific protein was detected with a goat anti-rabbit-HRP conjugated IgG Ab (Biorad, Richmond, CA.).

Immunohistochemistry: This was performed on 4T1 tumor tissue sections. For macrophage identification, biotinylated rat antimouse CD68 mAb was used with fluorescein-conjugated strepavidin as the secondary reporting reagent. Rabbit anti-Legumain antiserum was prepared by immunization with purified human Legumain produced in Escherichia coli. The reaction was visualized with Texas-red conjugated strepavidin, and the slides analyzed by laser scanning with a confocal microscope (Bio-Rad, Hercules, CA.). 4T1 tumor tissues were cut into sections and exposed to air until completely dry, fixed and stained for MMP-9, VEGF, TGF-β and F4/80 with the Rabbit immunoCruz^{TM} staining system (Santa Cruz, CA.), using rabbit anti murine MMP-9, VEGF, TGF-β mAbs (Santa Cruz, CA.) and F4/80 (Bioscience, CA.). This was followed by incubation at 4^{o}C overnight. A HRP-conjugated goat-anti-mouse secondary Ab was used and slides mounted with cells being visualized microscopically.

Vector construction, protein expression and transformation of S. typhimurium with DNA Vaccine Plasmids. Two constructs were made based on the vector pCMV (Invitrogen, San Diego, CA.), respectively. The pUb-Legumain construct was comprised of polyubiquitinated, full-length murine Legumain. The empty vector construct served as a control. Protein expression of Legumain was demonstrated by Western blotting with a polyclonal rabbit anti-murine Legumain Ab. Attenuated *Salmonella typhimurium* (dam^{-}; aroA^{-}) were transduced with DNA vaccine plasmids by electroporation as described in our previous publications [31,32].

Immunization and Tumor Cell Challenge. *Prophylactic model*: BALB/c or C57BL/6 mice were each divided into three experimental groups (n=8) and immunized with either empty vector or pUb-Legumain. All mice were challenged by i.v. injection with 5×10^4 CT-26 cells (BALB/c) or 2×10^5 D121 cells (C57BL/6), 1 wk after the last immunization, to induce either experimental or spontaneous pulmonary metastases. The survival rate of mice, lung weights and metastasis scores in experimental or control groups were determined. *Therapeutic model:* BALB/c mice were divided into three experimental groups (n=8) and first injected into the fat-pad with 7×10^3 4T1 cells on day 0 and then immunized 3 times with DNA vaccine starting on day 3 as described above. The experiment was terminated on day 28 to determine mouse lung weights.

In vivo depletion of $CD4^+$ or $CD8^+$ T cells, Cytotoxicity and ELISPOT assays. The depletion of $CD4^+$ or $CD8^+$ T cells in vivo was performed as previously described [33]. Cytotoxicity was measured and calculated by a standard ^{51}Cr-release assay as previously reported [34]. ELISPOT assays were performed with an ELISPOT kit (Pharmingen, La Jolla, CA.) according to instructions provided by the manufacturer.

Flow cytometry. Activation markers of T cells were measured by two-color flow cytometric analysis with a BD Biosciences FACScalibur. DC cell markers were determined by staining freshly isolated lymphocytes from successfully vaccinated mice and control mice with anti-CD11c Abs in combination with FITC –conjugated anti-CD40, CD80 and MHC-Class II Abs. Macrophages bearing high levels of $CD14^+$ and $F4/80^+$ were quantified by two-color flow analysis. Tumor cells were isolated from successfully vaccinated BALB/c mice and then stained with anti-CD14-APC, anti-F4/80-PE and anti-Legumain-FITC Abs, followed by FACS analyses. All antibodies were

purchased from PharMingen, San Diego, CA. IFN-γ release at the intracellular level was determined in lymphocytes of Peyer's Patches obtained 3 d after one time immunization and stained with APC- anti CD8 Ab. Cell were fixed, permeabilized and subsequently stained with PE-labeled anti-IFN-γ Ab to detect intracellular expression of IFN-γ.

Matrigel Assay. Matrigel was applied for evaluating the suppression of angiogenesis after vaccination. To this end, BALB/c mice were injected s.c. 2 wk after the last of 3 vaccinations, into the sternal region with growth factor-reduced Matrigel containing FGF-2 and 4T1 tumor cells ($7x10^3$) that were irradiated with 1000 Gy. The endothelium was stained 6 d thereafter by i.v. injection with *Bandiera simplofica* lectin I (Isolectin B4), conjugated with fluorescein (Vector Labs, Burlingame, CA.). This was done along with staining the endothelium of control animals, and 30 min later, mice are sacrificed, Matrigel plugs extracted and fluorescence evaluated by either fluorescence microscopy or fluorimetry.

Migration Assay. Cell migration assays were performed by using modified Boyden chambers (Transwell, Corning Inc., Corning, NY.). Unless indicated otherwise, cells were harvested by brief exposure to trypsin/EDTA (Invitrogen), followed by soybean trypsin inhibitor (Calbiochem). At the end of the assay, cells on the lower surface were fixed in 1% paraformaldehyde, stained with 1% crystal violet and counted [35].

Statistical Analysis. The statistical significance of differential findings between experimental groups and controls was determined by Student's *t* test, Findings were regarding as significant, if two tailed *P* values were <0.05.

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Competing Interests Statement

The authors declare that they have no competing financial interests.

Figure Legends

Figure 1. Legumain is highly expressed on Tumor Associated Macrophages (TAMs) in the tumor stroma

a. Legumain expression on TAMs was clearly evident as shown in Figure1a. In panel I, tumor-infiltrating macrophages are visualized by H/E staining as indicated by arrows. Legumain expression is indicated in panel II by double staining with anti-Legumain Ab (green) combined with anti-$CD68^{+}$ Ab (red).

b. Increased Legumain expression on TAMs was confirmed by Flow Cytometric analyses with double positive populations of $CD206^{+}/F4/80^{+}$- M2 macrophages that were isolated from fresh tumor tissue.

c. Multiple color flow cytometry demonstrated up-regulation of the M2 macrophage marker CD206 on RAW cells after being cultured with IL-4, IL-10 and IL-13 (10ng/ml). Furthermore, Legumain was shown to be highly expressed on $F4/80^{+}/CD206^{+}$ positive RAW cells cultured with IL-4, IL-10 and IL-13 as indicated in the upper panel when compared with $F4/80^{+}/CD206^{-}$ RAW cells depicted in the lower panel.

d. Confirmation of Legumain expression on RAW cells by Western blotting following stimulation with IL-13, IL-4 and IL-10, either singularly or combined.

Figure 2. Targeting of Legumain expressing cells results in suppression of tumor progression and metastases

a. Schematic of DNA vaccines constructed with the pCMV/myc/cyto vector backbone where the Legumain gene was fused to the C-terminal of mutant polyubiquitin. The entire fragment was inserted and protein expression was demonstrated by Western blotting.

b. Prophylactic model: The vaccination schedule was designed for three immunizations at 1-week intervals followed by an i.v. challenge with 2×10^5 D121 non-small cell lung cancer cells or $5x10^4$ CT26 colon cancer cells. Lung weights were determined 24 d after tumor challenge and analyzed in each group. Differences between the two control groups (PBS and empty vector) and the treatment group were statistically significant **$P<0.005$.

c. Therapeutic model: Groups of BALB/c mice (n=8) were initially injected into the mammary fat pad with 7 x 10^3 4T1 breast cancer cells and thereafter vaccinated three times on days 3, 7 and 11, and primary tumors excised on day 12. Survival plots represent results for 8 mice in each of the treatment and control groups.

d. Tumor metastasis scores and lung weights were measured 25 d after 4T1 tumor cell challenge, followed by 3 immunizations with either PBS, empty vector or the pLegumain vaccine, respectively. Results: metastasis scores are expressed as the % lung surface covered by fused metastatic foci: 0, none; 1, <5%; 2, 5% to 50%

and 3, >50%. Differences in lung weights between the group of mice treated with the Legumain vaccine and all control groups were statistically significant (**P<0.005). Normal lung weight is approximately 0.2g.

Figure 3. TAM population in the tumor stroma is decreased by $CD8^+$ specific CTLs induced by the Legumain-based DNA vaccine

a. RAW macrophage cells which highly express Legumain after culturing with 10ng/ml IL-4, IL-10, IL-13 served as target cells in a 4-hour ^{51}Cr-release assay. Splenocytes isolated from mice immunized with the pLegumain vaccine are shown to be effective in killing RAW cells treated with these cytokines *in vitro* at different effector-to-target cell ratios, but failed to induce cytotoxic killing of unstimulated RAW cells lacking Legumain expression. **P<0.005 compared to control groups.

b. Flow cytometry detects the percentage of TAM populations with specific macrophage markers—(CD206 and F4/80) in tumor tissue after vaccination. The percentage of TAM populations in tumor tissue cells, isolated from mice treated with our DNA vaccine, was shown to be reduced; however, there was no decrease in TAM populations isolated from mice treated with either empty vector or pLegumain or following $CD8^+$ T cell depletion (**P<0.005). In addition, TAM populations were slightly reduced, compared with controls, but not significantly (*P>0.05).

c. The results of Flow cytometry were confirmed by immunohistochemical staining with confocal microscopy. The population of TAMs in the tumor stroma was

dramatically decreased after vaccination. 4T1 cancer cells are shown in blue and TAMs in red. H/E staining (I); FITC-conjugated goat anti-rabbit IGg only, (II); pLegumain group, i.e. FITC-conjugated goat-rabbit plus rabbit anti-mouse Legumain Ab, (III); and empty vector group, stained like group III (IV).

Figure 4. MHC-class I restricted specific $CD8^+$ T cell response against Legumain expressing cells

a. DNA vaccination enhances expression of co-stimulatory molecules by DCs. Two color flow cytometric analyses were performed with single cell suspensions of lymphocytes obtained 3 d after vaccination. Lymphocytes from Peyer's Patches were stained with FITC labeled anti-CD11cAb in combination with either PE conjugated anti-CD80, MHC-I or CD40 Abs.

b. INF-γ release of $CD8^+$ T cells *in vivo* measured by FACS analysis. Splenocytes were obtained 3 d after immunization, stained with FITC-conjugated anti-CD8 mAb and fixed, permeabilized, and subsequently stained with PE-conjugated anti-IFN-γ Ab, and then analyzed by Flow cytometry. Bar graphs indicate mean and S.D. for four mice, **P<0.005, compared to control groups.

c. Production of INF-γ was verified *in vitro* at the single cell level by ELISPOT. This is indicated for single lymphocytes from immunized mice restimulated with either $Legumain^+$ 4T1 tumor tissue cells or $Legumain^-$ 4T1 cells, as determined by ELISPOT assay and depicted by the number of immunospots formed per well. Mean spot distribution for each well in each experimental and control group

(n=4). **P<0.005 compared to treatment group without stimulation. ##P<0.005 compared to control groups.

d. Splenocytes were isolated from vaccinated mice and analyzed for lytic activity in a 4-hour ^{51}Cr release assay with TAMs isolated from 4T1 tumor tissues serving as targets. Splenocytes isolated from mice immunized with pLegumain were effective in killing TAMs of 4T1 tumor tissue *in vitro* (*P<0.01, compared to control groups). The T cell-mediated killing was specific for Legumain because 4T1 cultured cells, lacking in Legumain expression, were not lysed. Inhibition experiments with Abs against $H2K^d$/$H\text{-}2D^d$ MHC class I antigen showed that the $CD8^+$ T cell mediated tumor lysis was MHC class I Ag restricted. Furthermore, *in vivo* depletion of $CD4^+$ or $CD8^+$ T cells indicate that lymphocytes isolated from vaccinated mice, that were thereafter depleted of $CD8^+$ T cells *in vivo,* failed to induce cytotoxic killing of tumor target cells. However, *in vivo* depletion of $CD4^+$ T cells did not abrogate cytotoxic killing of these same tumor target cells. *P<0.01 compared to PBS or empty vector group.

Figure 5. Reduction of TAMs results in a decrease in growth factor release, anti-angiogenesis and tumor cell migration

a. The Legumain-based DNA vaccine decreased the release of growth factors TGF-β, TNF-α and VEGF by TAMs. 4T1 breast tumor tissue and mouse serum were harvested on day 12 after vaccinations and used for tumor cell challenge in the mammary fat-pad. After 24h (VEGF, TGF-β) or 48h (TNF-α) culturing with RPMI1640, the supernatants of tumor tissue cells were harvested, and the

concentration of TGF-β, TNF-α and VEGF in both serum and supernatant measured by ELISA. There were statistically significant differences between the Legumain vaccine treatment group and control groups. *P<0.01, **P<0.005

b. Immunohistochemical staining by confocal microscopy. The biopsies of 4T1 tissues were stained to determine VEGF, TGF-β and MMP-9 expression in the tumor microenvironment. The pLegumain treated groups showed that VEGF, TGF-β and MMP-9 releases were decreased after a reduction in TAMs by the Legumain-based DNA vaccine, compared with empty vector groups. The two growth factors VEGF, TGF-β and MMP-9 are shown in green, and 4T1 breast cancer cells in blue.

c. Inhibition of VEGF-induced angiogenesis: BALB/c mice were vaccinated with either empty vector, pLegumain, or pLegumain after either $CD8^+$ or $CD4^+$ T cell depletion *in vivo*, respectively. Vascularization was induced by VEGF. Imaging and quantification of vessel growth were performed after *in vivo* staining of endothelium with FITC-labeled isolectin B4 and evaluation by fluorimetry. There was a decrease in the VEGF-induced neovasculature only after vaccination with the vector encoding Legumain but not after vaccination with the empty vector or with pLegumain after depletion of $CD8^+$ T cells. **P<0.005, *P<0.01 compared to the Legumain treatment group.

d. A transwell migration assay was performed to determine tumor cell migration and invasion abilities after vaccination. The number of migrating cells was markedly reduced after vaccination with the Legumain based-DNA vaccine. ***P<0.001 compared to the empty vector group.

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Figure 1. Legumain is highly expressed on Tumor Associated Macrophages in tumor stroma

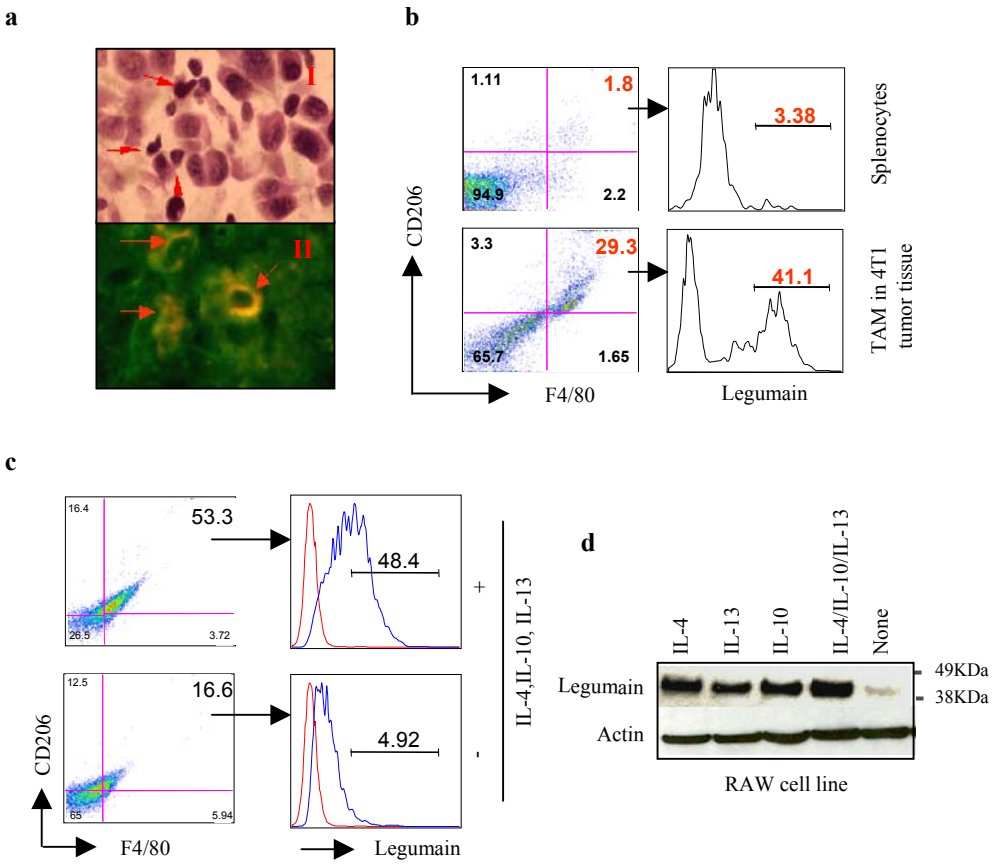


Figure 2. Targeting Legumain expressing cells results in suppression of tumor progress and metastases

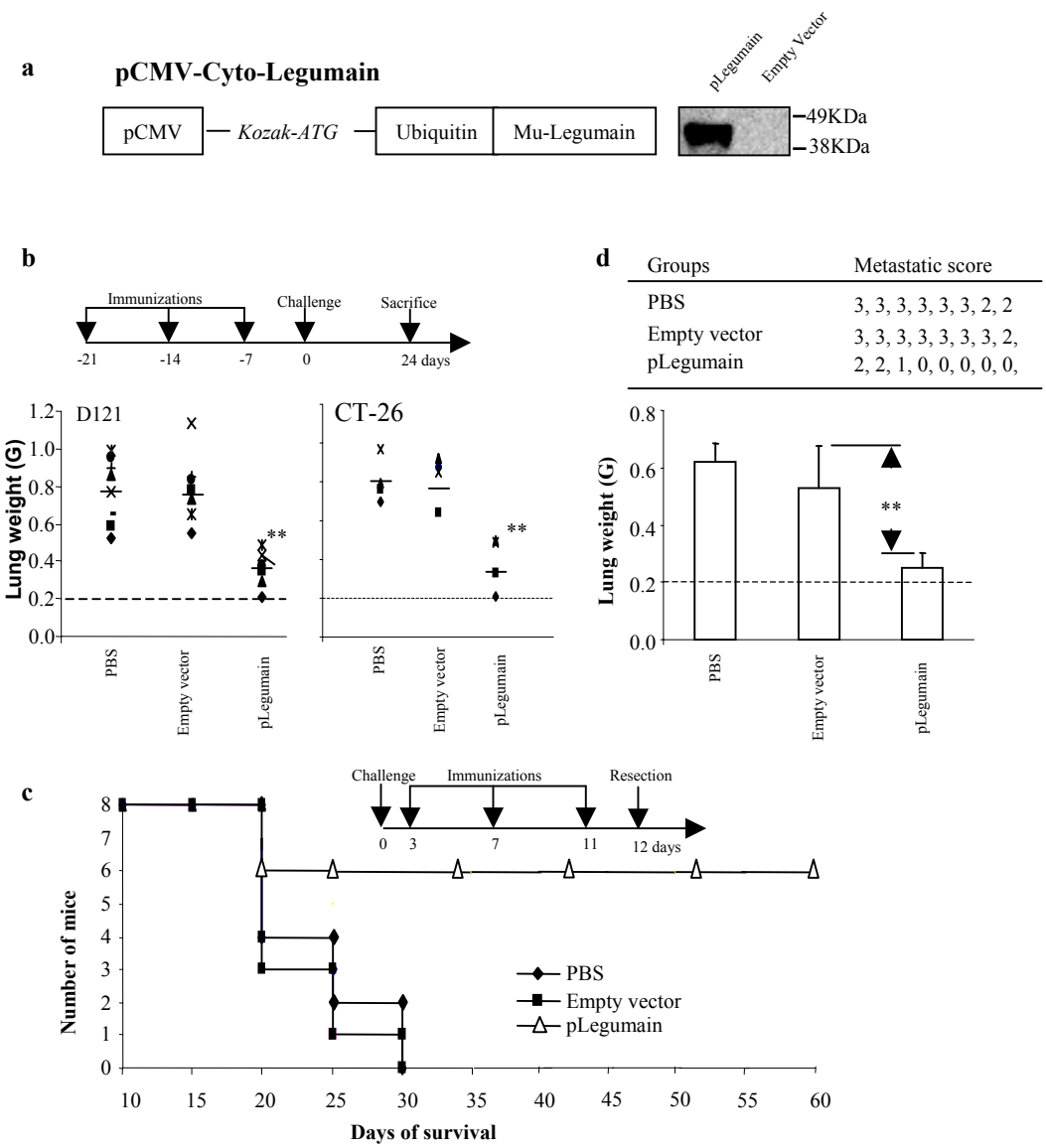


Figure 3. Decreased TAM population by $CD8^+$ specific CTL induced via pLegumain DNA vaccine

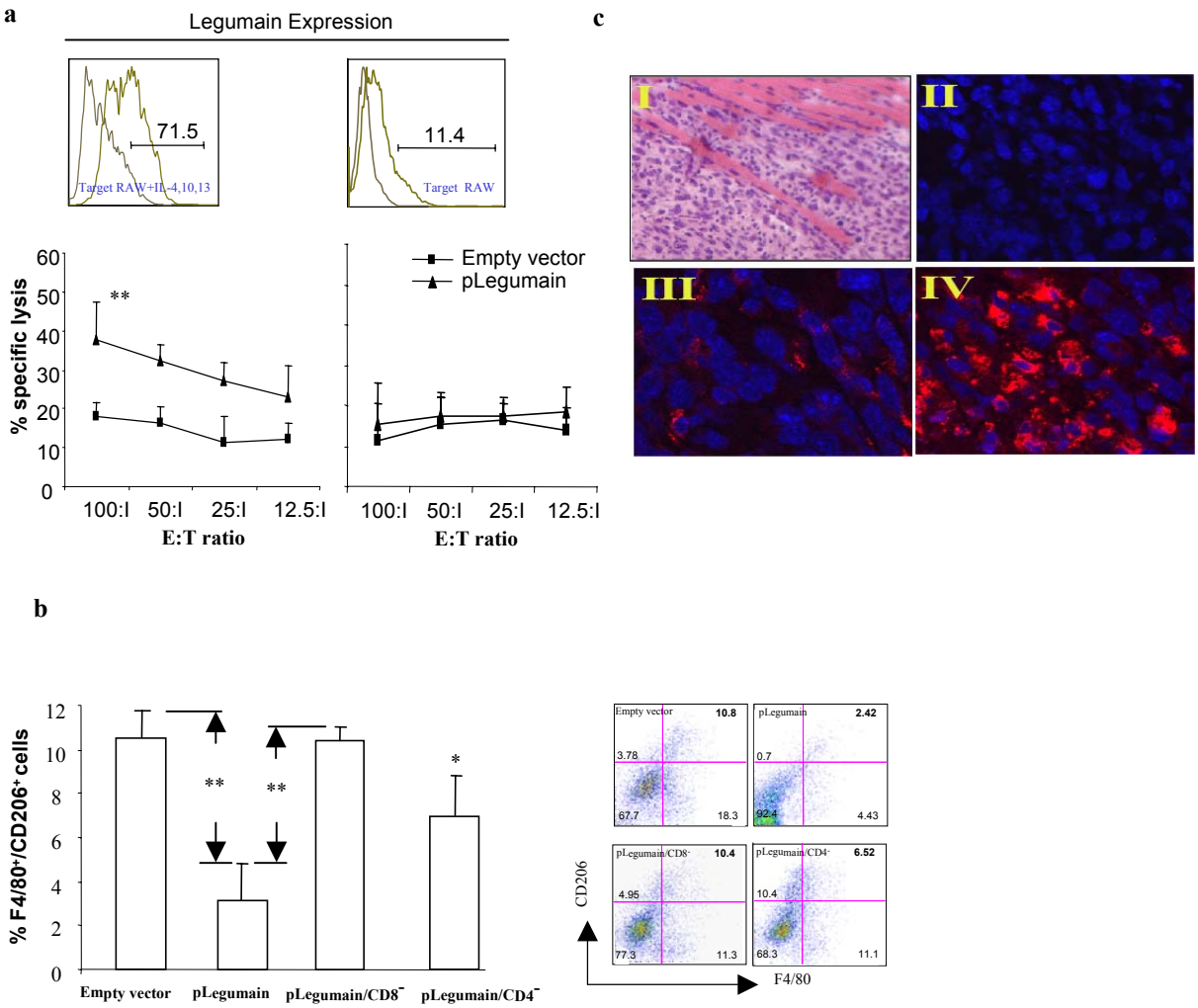


Figure 4 MHC-class I restricted specific CD8$^+$ T cell response against Legumain expressing cells induced by Legumain based DNA vaccine

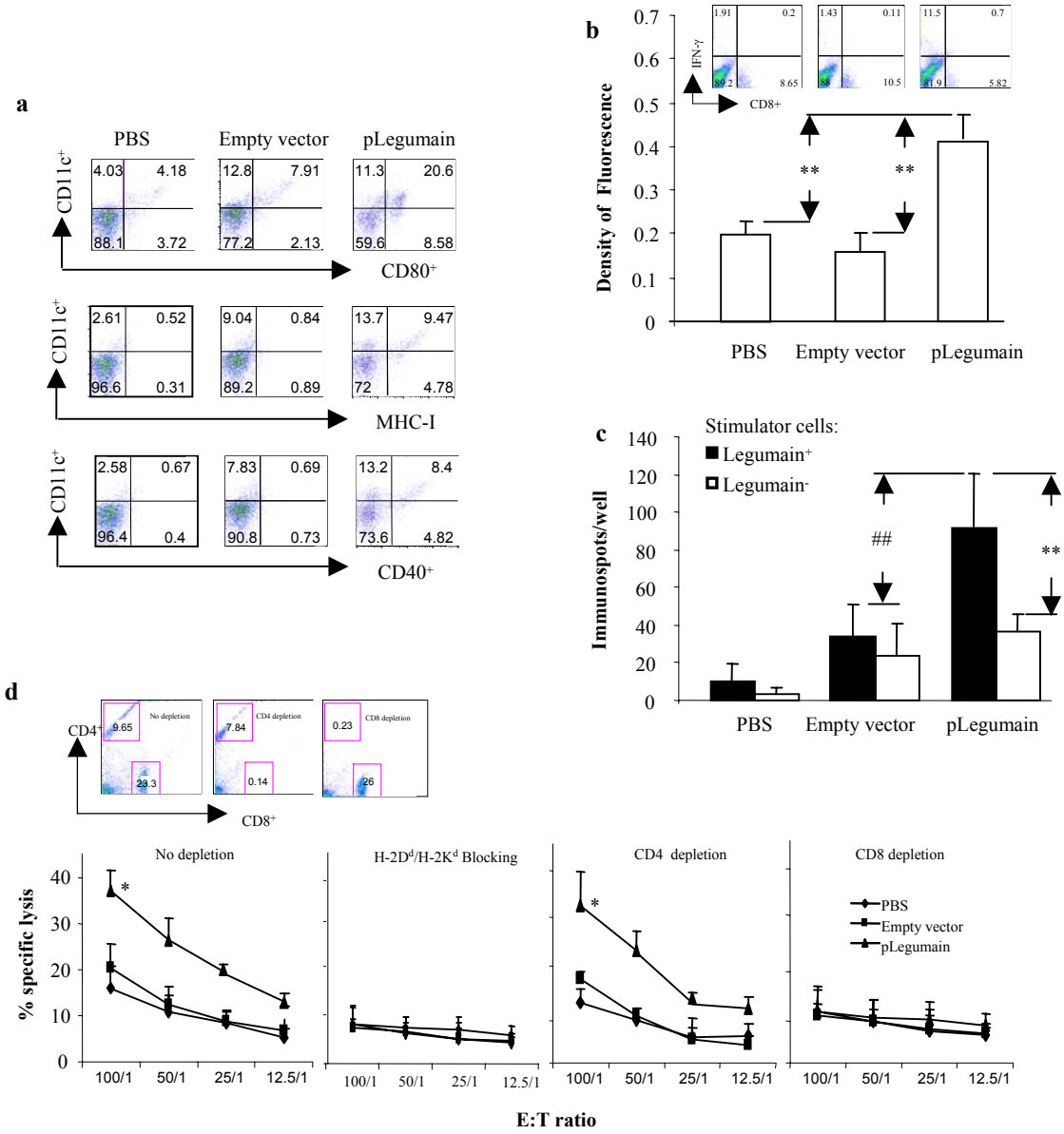
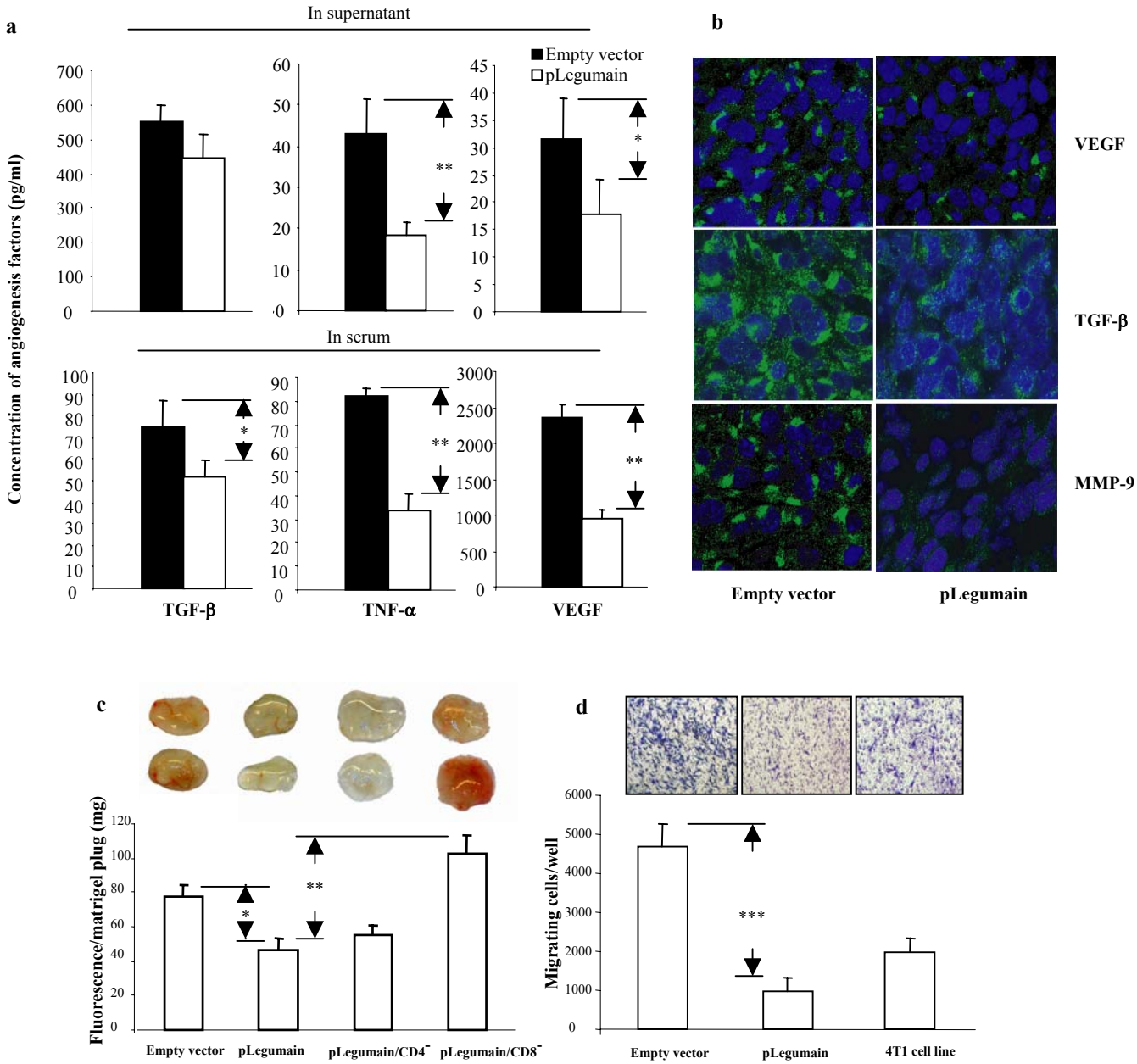


Figure 5. Abrogation of TAMs results in decreasing growth factors release, anti-angiogenesis and tumor cell migration



Appendix II: Abstracts

12. Yunping Luo, He Zhou, Masato Mizutani, Noriko Mizutani, Ralph A, Reisfeld and Rong Xiang. 2003. Targeting of transcription factor Fos-related antigen 1 induces a long-lived protective memory T cell response against breast cancer. AACR 94th Annual Meeting, Washing, D.C. (Poster presentation)
13. He Zhou, Yunping Luo, Masato Mizutani, Noriko Mizutani, Carrie Dolman, Dorothy Markowitz, Rong Xiang, Ralph A Reisfeld. 2004. A DNA minigene vaccine against VEGF receptor 2 (Flk-1) suppresses angiogenesis and successfully inhibits growth of prostate and lung carcinoma in mice. AACR 95th Annual Meeting, Orlando, FL. (Poster discussion presentation)
14. Noriko Mizutani, Masato Mizutani, Yunping Luo, He Zhou, Ralph A. Reisfeld, and Rong Xiang. 2004. A DNA vaccine encoding endoglin suppresses angiogenesis and growth of breast cancer. AACR 95th Annual Meeting, Orlando, FL. (Poster presentation)
15. Yunping Luo, He Zhou, Chen Liu, Rong Xiang and Ralph A. Reisfeld. 2005. A novel stress protein, legumain, is a target for a genetic vaccine against breast cancer. AACR 96th Annual Meeting, Anaheim, CA. (Poster presentation)
16. He Zhou, Yunping Luo, Jeng-fan Lo, Masato Mizutani, Noriko Mizutani, Rong Xiang, and Ralph A. Reisfeld. 2005. A DNA-based Cancer vaccine activates both innate and adaptive immunity by engaging the NKG2D receptor. AACR 96th Annual Meeting, Anaheim, CA. (Poster presentation)
17. Yunping Luo, He Zhou, Joerg Krueger, Charles Kaplan, Carrie Dolman, Dorothy Markowitz, Wenyuan Wu, Cheng Liu, Ralph A. Reisfeld and Rong Xiang. 2006. Targeting Tumor-Associated Macrophages: A Novel Strategy against Breast Cancer. AACR 97th Annual Meeting, Washing, D.C. (Poster presentation)
18. He Zhou, Yunping Luo, Charles D. Kaplan, Jörg A. Krüger, Sung-Hyung Lee, Rong Xiang, and Ralph A. Reisfeld. 2006.A DNA-based cancer vaccine enhances lymphocyte crosstalk by engaging the NKG2D receptor. AACR 97th Annual Meeting, Washing, D.C. (Oral presentation)

Targeting of transcription factor Fos-related antigen 1 induces a long-lived protective memory T cell response against breast cancer

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Abstract

A novel approach successfully induced breast tumor-specific immunity, followed by a long-lived T-memory cell response resulting in protection against breast cancer metastasis. We achieved this goal by targeting Fos-related transcription factor Fra-1 with an oral DNA vaccine delivered by attenuated *Salmonella typhimurium* to secondary lymphoid organs and boosted by co-expression of secretory cytokine IL-18. Our data revealed not only the successful induction of a $CD8^+$ T cell-mediated antitumor response, but also a long-lived memory T cell response against breast carcinoma in both syngenic and SCID mice, following an initial tumor cell challenge. Importantly, we demonstrated for the first time a mechanism whereby IL-18 plays a key role in the maintenance but not the induction of T cell memory. Furthermore, we also found long-lived memory T cells to be dormant in non-lymphoid tissues following an initial D2F2 breast tumor challenge. Significantly, these T cells were highly cytotoxic and quickly rebounded again following a second tumor cell challenge. This is in comparison to $CD8^+$ T cells found in secondary lymphoid tissues which also maintain a long-lived memory T cell response. Our immunotherapeutic approach could be useful in the general setting of weakly immunogenic tumor-self antigens to induce a long-lived and potent immune memory response against cancer.

A DNA minigene vaccine against VEGF receptor 2 (Flk-1) suppresses angiogenesis and successfully inhibits growth of prostate and lung carcinoma in mice

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Tumor cells are elusive targets for immunotherapy due to their heterogeneity and genetic instability. Our previous work showed a DNA vaccine encoding the entire Flk-1 gene can successfully protect mice from lethal challenges with melanoma, colon carcinoma and lung carcinoma cells via a tumor vasculature-specific CTL response and subsequent suppression of angiogenesis. Here, a minigene approach was used in an attempt to improve efficacy of such vaccines, identify CTL epitopes and study the relevant mechanism(s) involved. Three nonapeptides, namely Flk_{94}-RVVGNDTGA, Flk_{400}-VILTNPISM, and Flk_{1210}-FHYDNTAGI, were selected for the minigene vaccine based on the prediction of H-2 Db binding affinity. A pCMV plasmid containing a HIVtat translocation peptide was used as the vector for efficient delivery and processing of the peptides in the ER. Mice were immunized orally with attenuated *Salmonella typhimurium* harboring the Flk-1 minigene vector or empty vectors, 3 times at 1-week intervals and challenged with tumor cells 2 weeks after the last immunization. We demonstrate that this minigene vaccine successfully protects mice from lethal injection of lung carcinoma-D121 and prostate carcinoma-RM9, resulting in improved survival rates and fewer metastatic foci. Compared to groups vaccinated with empty vectors, splenocytes isolated from minigene-vaccinated mice showed greater killing of endothelial cell lines expressing Flk-1, to an extent very similar to that achieved by splenocytes isolated from mice vaccinated with the entire Flk-1 gene. This killing seemed to be specific since only low level of killing of Flk-negative RM9 cells was observed in all the experimental groups. Splenocytes stimulated with Flk_{400} or Flk_{1210} peptides showed specific killing of Flk-1 expressing endothelial cells. However, unstimulated splenocytes or splenocytes stimulated with Flk_{94} exhibited non-specific killing, i.e. similar killing of both Flk-1 positive endothelial cells and Flk-1 negative RM9 tumor cells. These data suggest Flk_{400} and Flk_{1210} to be H-2 Db restricted specific CTL epitopes. Overall, our data demonstrate that Flk-1 minigene vaccines can achieve anti-angiogenesis by specifically inducing a Flk-1-specific CTL response against endothelial cells rather than tumor cells, thereby protecting mice from lethal challenges with prostate and lung carcinoma cells.

A DNA vaccine encoding endoglin suppresses angiogenesis and growth of breast cancer

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Endoglin is a transforming growth factor-binding protein, which is overexpressed on the surface of proliferating endothelial cells undergoing angiogenesis. The latter plays a key role in the growth and dissemination of solid tumors and is a rate-limiting step in their development of solid tumors. Therefore, we developed a novel genetic approach to breast cancer with an oral DNA vaccine encoding the entire endoglin gene, carried and delivered to secondary lymphoid tissues by attenuated *Salmonella typhimurium*. Here, we demonstrate that growth of primary subcutaneous breast tumors and dissemination of their pulmonary metastases is markedly suppressed by this oral DNA vaccine. Lifespan of all vaccinated mice (n=8) was doubled after lethal tumor cell challenge in the absence of detectable tumor growth. Immunological mechanisms involved activation of T- and dendritic cells as indicated by upregulation of their activation markers and costimulatory molecules. Markedly increased specific target cell lysis was mediated by MHC class I-restricted $CD8^+$ T cells isolated from splenocytes of vaccinated mice, including a significant release of proinflammatory cytokines IFN-γ and IL-2. Most important, analysis of basic fibroblast growth factor-2 and tumor cell-induced vessel growth in Matrigel plugs demonstrated marked suppression of angiogenesis only in vaccinated animals. Taken together, this endoglin-based DNA vaccine provided a new strategy for Ralph A. Reisfeld protecting against growth and metastases of murine D2F2 breast cancer cells by combining the action of immune effector cells with suppression of tumor angiogenesis.

A novel stress protein, legumain, is a target for a genetic vaccine against breast cancer

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Legumain, an asparaginyl endopeptidase functioning as an overexpressed stress protein on mammary adenocarcinoma, proliferating endothelial cells and tumor-associated macrophages (TAMs), can serve as an effective target for DNA-based vaccines to induce sufficiently robust T-cell-mediated and humoral immune responses. We report here some of the evidence obtained thus far in our experiments. First, we demonstrated that legumain was over-expressed *in vivo* on most of the solid tumors tested, especially on neoplastic cells, neovasculature and TAMs, but not by corresponding tumor cell lines in tissue culture. Importantly, most normal tissues reveal either none essentially undetectable levels of legumain expression. Second, legumain is a stress-responsive protein induced under certain conditions such as heat shock, drug treatment, and hypoxia and is associated with tumor invasion, dissemination of metastases and tumor angiogenesis. Third, our results indicate that our legumain-based DNA vaccine with co-expressing secretory chemokins induced suppression of both 4T1 primary breast tumor growth and dissemination of their spontaneous pulmonary metastases in a prophylactic setting. Finally, this anti-tumor effect can be achieved by suppression of angiogenesis in the tumor vasculature combined with CTL-mediated and/or Ab-mediated tumor cell killing. Taken together, this newly discovered stress protein legumain serves as an effective vaccine target against breast cancer with an approach that may lead to the rational design of vaccines for clinical application.

A DNA-based Cancer vaccine activates both innate and adaptive immunity by engaging the NKG2D receptor

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The natural killer cell receptor NKG2D mediates stimulatory or co-stimulatory signals to NK cells and $CD8^+$ T cells, respectively. We hypothesize that by engaging the NKG2D receptor, DNA-based cancer vaccines can achieve better activation of both innate and adaptive immune response, and therefore lead to better vaccine efficacy. We tested this hypothesis in a survivin-based DNA vaccine. Survivin is an inhibitor of apoptosis protein and is over-expressed in essentially all solid tumors. Mice were immunized orally twice at a 2-wk interval with attenuated *Salmonella typhimurium* (*AroA*$^-$, *dam*$^-$) harboring expression vectors encoding H60, one of the natural ligands of NKG2D receptor, or Survivin or both H60 and Survivin. Mice were challenged with tumor cells 2 wk after the second vaccination in a prophylactic setting, or 5 d before the first vaccination in a therapeutic setting. Two different tumor models were used, either CT-26 colon carcinoma cells, which express NKG2D ligands at a minimal level or D2F2 breast carcinoma cells expressing these ligands at intermediate level. Here, we demonstrate that the introduction of H60 in conjunction with survivin in a DNA-based vaccine proved to be superior in protecting mice from challenges with tumor cells of different origin and different NKG2D ligand expression levels, both in prophylactic and therapeutic settings. This protection seems to depend on CD8 T- and NK cells, since their depletion diminished protection against tumor cell challenges. The H60/Survivin DNA vaccine induced enhanced NK activity as demonstrated by cytotoxicity assays against Yac-1 targets. Mice vaccinated with H60/Survivin also exhibited greater CTL activity against CT-26 tumor cells, and this cytotoxicity was inhibited by antibodies against CD8 or MHC class I Ag, but not by antibody against CD4. Crosstalks also exist between CD8 T cells and NK cells since depletion of CD8 cells impaired NK activity, while depletion of NK cells led to reduced CTL activity. The H60/survivin DNA vaccine induced both robust activation of dendritic cells and changes in homing of different lymphocyte populations to Peyer's Patches, indicating that changes in the microenvironment inside such secondary lymphoid tissues induced by H60/Survivin vaccination favor NK cell activation and CD8 T cell priming. Taken together, our observations demonstrate that NKG2D ligands, such as H60, activate both innate and adaptive immunity resulting in more effective DNA-based cancer vaccines.

Targeting Tumor-Associated Macrophages: A Novel Strategy against Breast Cancer

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Tumor development and progression are strongly linked to inflammation and the presence of tumor-associated macrophages (TAMs). Thus, antitumor activity can be achieved by targeting TAM recruitment, activation and extracellular matrix interactions. TAMs consist primarily of a polarized M2 ($CD206^+$, $F4/80^+$) macrophage population with little cytotoxicity for tumor cells and poor antigen presenting capacity. Legumain, a member of the asparaginyl endopeptidase family, functioning as a stress protein, was found highly up-regulated on TAMs in many murine and human tumor tissues, but absent or only present at very low levels in all normal tissues and on circulating macrophages of M1 phenotype. Here, we demonstrate for the first time that the intense overexpression of Legumain on TAMs provides for a unique and novel approach to tumor therapy. In fact, immunization against Legumain dramatically reduced the density of TAMs in tumor tissues, resulting in a decrease of pro-angiogenesis as well as tumor growth factors released by these macrophages. This, in turn, decisively suppressed tumor angiogenesis and tumor growth and metastasis. Importantly, we demonstrated the success of this anti-TAM strategy in three different murine tumor models, in either prophylactic or therapeutic settings. In conclusion, reducing the density of TAMs in the tumor stroma sufficiently remodeled the tumor microenvironment and allowed to gain further insights into mechanisms required for an effective intervention in tumor growth and metastasis that may ultimately lead to better anticancer strategies.

A DNA-based cancer vaccine enhances lymphocyte crosstalk by engaging the NKG2D receptor

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The NKG2D receptor is a stimulatory receptor expressed on NK cells and activated CD8 T cells. We demonstrate that engaging the NKG2D receptor markedly improves the efficacy of a survivin-based DNA vaccine. Survivin is an inhibitor of apoptosis protein and is over-expressed in essentially all solid tumors. The combination vaccine encoding both a NKG2D ligand H60 and survivin activates both innate and adaptive antitumor immunity, resulting in better protection against tumors of different origin and NKG2D expression levels. The enhanced vaccine efficacy is in part due to increased crosstalk between lymphocytes. Depletion of either CD8 T cells or NK cells during priming diminishes the pH60/Survivin-induced activation of dendritic cells (DCs) in Peyer's patches. More importantly, depletion of CD8 T cells or NK cells during the priming, but not the effector phase of immunization, leads to reduced NK or CTL activity, respectively. In contrast, depletion of CD4 T cells during the priming phase results in activation of DCs, NK and CD8 T cells in Peyer's patches and enhancement of NK cell activity. These data suggest NK and CD8 T cells as positive contributors and CD4 T cells mainly as negative regulators during the lymphocyte crosstalk induced by our DNA vaccine. Vaccination with pH60/Survivin reduces the number of CD4 T cells, but increase that of DC and NK cells which home to Peyer's patches. Presumably this is due to changes in homing receptor profiles of these cells. Thus, by preferentially activating and attracting positive regulators and reducing negative regulators in these secondary lymphoid tissues, our dual function DNA vaccine induces a microenvironment more suitable for NK cell activation and T cell priming. As a result, the combination vaccine induces a robust activation of lymphocytes in Peyer's patches. The effectiveness of our strategy is demonstrated by better tumor protection in both prophylactic and therapeutic settings and long-lived immune memory against tumor cells, suggesting that activation of both the innate and adaptive arms of the immune system represents an attractive strategy to overcome tumor-induced peripheral immune tolerance.